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The evaluation of behavioural tasks and animal models of Alzheimer's disease for assessing putative cognition enhancers, using a cholinesterase inhibitor as reference compound

The evaluation of behavioural tasks and animal models of Alzheimer's disease for assessing putative cognition enhancers, using a cholinesterase inhibitor as reference compound

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ABSTRACT

Metrifonate is a second generation cholinesterase inhibitor (ChEI) and was used in this study as a reference compound to evaluate behavioural tasks and models that could be employed to test future putative cognition enhancers in the treatment of Alzheimer's Disease, a neurodegenerative disorder which is characterised by a decline in memory and other cognitive abilities.

- The biochemical activity of metrifonate was assessed, specifically in relation to the development of tolerance after repeated exposure to the compound. With administration of central or peripherally acting cholinergic compounds to animals pre-treated with metrifonate it was found that the development of tolerance is a centrally acting phenomenon and specifically related to the cholinesterase molecule. Further study on the gene expression of acetylcholinesterase and butyrylcholinesterase showed that there is a two fold up regulation of the gene increasing the amount the these two proteins. This up regulation of ChE expression could be responsible for the decrease in adverse side effects observed over time.

- Metrifonate was observed after either chronic or acute administration in the passive avoidance task that is commonly used as a fast screening tool. Acute metrifonate had no effect on the performance deficits in the retention session. The chronic study demonstrated that the reference compound effectively inhibited the scopolamine-induced deficit.

- Sub-chronic metrifonate was tested in neurologically normal rats in the standard Morris water escape task and failed to improve spatial memory. Metrifonate had no ameliorating effects on water maze navigation in mice with scopolamine-induced amnesia, though metrifonate improve spatial performance of rats with bilateral lesions of the entorhinal cortex (EC) suggesting that under conditions of pathological impairment of brain structures such as entorhinal cortex lesion, rather than pharmacological intervention such as scopolamine, future ChE-Is might produce beneficial effects on learning and memory.

- The Peak interval procedure was used to assess the effects of the ChEI's on time estimation. A single administration Metrifonate (60 mg/kg) shifted the peak time to the right, indicating an impairment in time estimation, whereas sub-chronic administration of metrifonate had no effect on time estimation.

- Sub chronic metrifonate treatment (60 mg/kg) increased the escape latency to reach the platform. However there was no difference in choice accuracy. No specific conclusion could be drawn as to the effectiveness of this task in assessing future cognition enhancers

Although animal models used in this thesis, particularly EC lesions, rats show, mnemonic deficits similar to those seen in AD, the relationship is less than perfect. From this study it is recommended that the efficacy of a test compound should be determined in a series of tests that cover a variety of cognitive processes. Employing animal models that integrate lesions, transgenics and/or pharmacological manipulation could increase the validity of animal models of behavioural dysfunctions and therefore the evaluation of future cognition enhancers could become more effective.

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Author's Declaration

I declare that the work in this dissertation was carried out in accordance with the regulations of the University of Bristol. The work is original except where indicated by special reference in the text and no part of the dissertation has been submitted for any other degree.

Any views expressed in the dissertation are those of the author and in no way represent those of the University.

The Dissertation has not been presented to any other University for examination either in the United Kingdom or overseas.

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GENERAL INTRODUCTION

1.1 Alzheimer's Disease

The term cognition refers to all processes by which sensory inputs from an environmental stimulus are perceived; how association between stimuli are compared, stored, and then used to modify behaviour on the basis of past experiences. Learning efficiency depends upon factors such as motivation, attention, opportunity for rehearsal and prior experience (D'Mello et al 1996).

Conditions that disrupt cognitive function are devastating. For example Parkinson's disease, Huntingdon's Chorea and Schizophrenia are often accompanied by cognitive deficits. Normal aging is associated with a decline in functions such as sensory and motor performance and at times this decline is accompanied by declining cognitive function. Dementia is a group of conditions that is characterised by a gradual deterioration of intellectual abilities to the point that social and occupational functions are impaired. Alzheimer's disease (AD) is the form of dementia that is produced by disease directly attacking the brain. This is a progressive clinical state characterised by deterioration in intellect, memory, judgment and abstract thinking (American Psychiatric Association: DSM IV, 1994). Patients afflicted with cognitive disorders feel they no longer have a good quality of life and eventually may require full time care for they are unable to function independently. AD effects about five percent of the population older than 60 years of age (Coyle et al, 1983; Katzman, 1986) and in the United States can account for around 100,000 deaths per year (Robertson, 1990). As the average life span increases, with a greater percentage of an aging population in industrialised western countries, it is expected that a dramatic increase of AD will follow in the near future (Brody, 1990). The prevalence of AD doubles rapidly every 4.2 years (Molnar et al, 1997).

AD is occasionally inherited within families in which case it is called familial AD, and this usually has an early onset between the ages of 35 and 55. It can be distinguished from nonfamilial or sporadic AD, which generally has a later onset (approximately 60 to 65 years). The familial forms of AD can be traced to several genetic mutations.

Symptomatology

The syndrome typically begins with the onset of *amnesia* (a selective impairment in memory) and subsequently includes elements of *aphasia* (impairments in naming and the use of vague and empty speech), *apraxia* (impairments in the performance of routine tasks like cooking and dressing) and *agnosia* (inability to recognise objects and familiar faces). A problem with executive function includes impairments in judgement, reasoning and the ability to plan, execute, monitor and revise certain activities. In advanced stages of the illness, individuals are severely confused, bedridden, incontinent and unable to feed themselves. The duration of the illness is highly variable, though it commonly lasts 8-10 years.

In addition to their cognitive impairments most patients develop other behavioural disturbances. These include suspiciousness, paranoia, delusions, hallucination, wandering and pacing verbal and physical aggression, apathy and social withdrawal, repetitive behaviours and depression.

Hasegawa and Aoba (1994) proposed 3 stages of increasing intellectual impairment in AD.

- Stage 1: - the amnesia stage. This corresponds to the early clinical symptoms of AD, which are typically manifested as short-term memory deficits. They may last 2-4 years before progressing to the next stage.
- Stage 2: - the confusional stage. This involves a continuing decline in cognitive functioning, which also might include severe mental confusion, personality changes and behaviour disturbances such as extreme aggressiveness or wandering and sometimes psychotic episodes.
- Stage 3: - the dementia stage. This involves complete dementia in which the patient is withdrawn, unresponsive, incontinent and unable to care for him or herself. Ultimately the patient is likely to die from a secondary illness or problem such as bronchopneumonia, urinary infection or aspiration.

Neuropathology

Alzheimer's disease is characterised by several distinct neuropathological features: neuritic (senile) plaques and neurofibrillary tangles and loss of neurons and synapses (Terry et al, 1994; Hof et al, 1994).

Senile plaques are spherical, multicellular lesions that are usually found in moderate or large numbers in limbic structures (Dickson, 1997). They contain cellular deposits of ß-amyloid protein (Aß), a peptide made up of a varying number of amino acids (42-43; Zubenko, 1997). It is synthesised from a larger protein called the ß-amyloid precursor protein (APP). APP has shown to be coded by a single gene (Goldgaber, 1987).

Neurofibrillary tangles are intraneuronal cytoplasmic lesions consisting of non-membrane bound bundles of paired or helically wound filaments (PHF; Goedert *et al*, 1996). The tangles generally occur in large numbers in the entorhinal cortex, hippocampus, amygdala, frontal, temporal, and parietal lobes. The subunit protein of the PHF is the microtubule-associated protein, Tau (Selkoe, 1991). It is found to be hyperphosphorylated and highly insoluble and promotes dissociation of the protein from microtubules and self-aggregation into the PHFs in the neurofibrillary tangles (Selkoe, 1999).

The major histopathological features of AD probably contribute to cognitive decline, neuritic plaques appear to form in the early stages of AD (Morris et al, 1996) however, they are not correlated to the severity of cognitive impairment (Bierer et al, 1995). In contrast, neurofibrillary tangles appear to develop in conjunction with the progression of the illness (Gomez-Lima at al, 1997). Loss of synapses and neurons is very strongly correlated with the severity of cognitive decline (Gomez-Lima at al, 1997).

A massive decrease of basal forebrain cholinergic neurones (particularly in the nucleus basalis of Meynert, NBM; Bowen *et al* 1976; Whitehouse et al, 1982) is paralleled by large reductions in cholinergic markers such as Choline acetyltransferase (ChAT, Perry et al, 1978; Reisine et al, 1978), reduced level of acetylcholine (Ach, Bowen et al, 1988) and reduced muscarinic and nicotinic receptor binding (Mash et al, 1985; Whithouse et al, 1987, Giacobini, 1989). These markers of cholinergic denervation are highly correlated with the degree of dementia and neuropathological changes in AD (Perry et al, 1978; Bierer et al, 1995). The presumed causal relationship between the pronounced decline in cholinergic activity and cognitive dysfunction led Bartus and co-workers to introduce their 'cholinergic hypothesis of geriatric memory dysfunction' (Bartus et al, 1982).

There are indications that other neurotransmitter systems may also be compromised in AD. Noradrenergic deficits have been found in the cortex and in the hypothalamus (Rossor and Iversen, 1986). In the cortex there is some loss of both presynaptic 5-HT concentrations and uptake sites and post-synaptic 5-HT_1 and 5- HT_2 receptors (Court and Perry, 1991). It has also been reported that there is a significant loss of cortical somatostatin neurones and receptors in AD (Geula and Mesulam, 1994; Rossor and Iversen, 1986). Cholinergic fibres innervate cortical somatostatin neurons and stimulate somatostatin release. Therefore it might be possible that somatostatin neurons may be damaged due to the loss in cholinergic innervation.

Genetics

There are four well-confirmed genes in which mutations or polymorphisms occur that can result in AD. The first AD causing gene to be identified was that encoding the precursor Aß, the ß-amyloid precursor protein (APP) that is located on chromosome 21 (St George-Hyslop *et al*, 1987). Mutations of the APP gene can cause familial forms of AD. APP in cell membranes is processed by proteolytic breakdown that gives rise to various fragments of the molecule. The initial cleavage of APP is accomplished by a protease, α- secretase (review; Checler, 1995). The site of action of this protease is within the Aß domain of APP. This results in the release of soluble APP. Another less frequent proteolytic pathway involves the recently identified ß-secretase (Vassar *et al*, 1999; Hussain *et al*, 1999) and the still unidentified γ-secretase. In the hypothesised processing pathway necessary for amyloid deposition, a protease cleaves APP. Subsequent action of γ-secretase liberates the fibrillogenic and potentially neurotoxic $Aß_{42-43}$ peptide, which is found in AD senile plaques.

The AD1 locus on chromosome 19 was discovered to be associated with differential risk for late-onset familial and sporadic AD (van Broekhaven, 1995). The critical gene within this locus was discovered to be *apolipoprotein E* (apoE*;* Corder *et al*, 1993). There are three major alleles of the apoE gene, designated apoE2, apoE3 and apoE4 and each individual possesses two of the alleles of the apoE gene. It has been shown that the apoE4 heightens the risk for late-onset AD; whereas apoE2 exerts somewhat of a protective effect i.e. the individual is likely to develop the disease later in life. There is evidence for a role of apoE in amyloid deposition and Aß fibril formation (Namba *et al*, 1991; Kida *et al*, 1994).

Sherrington and colleagues (1995) identified a gene within the AD3 locus on chromosome 14, which, when mutated, appears to be linked to early onset familial AD with the age of onset being between 35 and 55 years. The gene was initially termed S182 and was subsequently given the name *presenilin-1* (PS-1). The function of the PS-1 protein is unknown, however putative functions for PS1 include roles in the regulation of signal transduction during development, in apoptosis and possibly in cellular calcium ion homeostasis (Fraser *et al*, 2000). A novel locus for a similar gene on chromosome 1 has been named *presenilin-2* (PS-2; Levy-Lahad *et al*, 1995). The PS-2 protein shows significant sequence homology to PS-1, and when mutated is also thought to be linked to AD, though the age of onset of the disease in patients carrying PS-2 ranges from 40 and 85 years. Both proteins reside within the endoplasmic reticulum, Golgi and nuclear envelope. Given the strong similarities in structure and amino acid sequence, it is likely that PS-1 and PS-2 have similar or overlapping activities (Fraser *et al*, 2000).

In summary, early onset forms of familial AD show patterns of simple autosomally dominant inheritance that have been linked to mutations in three different genes; APP, PS-1 and PS-2. These mutations are thought to directly (APP) or indirectly (PS-1 and PS-2) affect APP processing, Aß deposition and amyloid plaque formation.

Variation in the apoE4 gene has been shown to influence the risk for and severity of, late-onset familial and sporadic AD (for a recent review see: Van Gassen and Van Broeckhoven 2000). For a general view on age of onset, the chromosome and gene involved and the genetic effect, see table 2.

Table 2: Genetic factors in Alzheimer's disease. Summarised are the age of onset, the chromosome and gene involved and the genetic effect in Alzheimer's disease.

Type	Chromosome	Gene	Genetic Effect
Early- Onset, Familial	21	APP	Direct Linkage
Early- Onset, Familial	14	PS-1	Direct Linkage
Early- Onset, Familial	1	PS-2	Direct Linkage
Late - Onset, Familial and sporadic	19	APOE	Risk Factor

1.2 The cholinergic system

Acetylcholine: Synthesis

Acetylcholine (Ach) was first synthesised in 1837 though its biological importance was not discovered until much later. It was not until 1921 that Loewi, a German pharmacologist, established the role of acetylcholine as a neurotransmitter in the heart of a frog. Stimulation of the Vagus nerve induced a drop in heartbeat. Simultaneously, the heart was perfused with physiological solution, which subsequently led through a second denervated heart. He observed that the stimulation induced drop in heartbeat also occurred in the recipient heart. Loewi called the substance "*Vagusstoff*". He did not realise that this was actually Ach. In 1914, Dale noted that applying Ach to the nerves mimicked the effects of stimulating the parasympathetic fibres.

Many subsequent experiments revealed similarities between Ach and Vagusstoff, however they were not accepted as being identical, because Ach had not been shown to be a constituent of the body. Ach was subsequently isolated from spleen extracts and the parallel actions of Ach and Vagusstoff finally led to acceptance that they were the same substance (review; Karczmar, 1993). Later it was shown that Ach was also the neurotransmitter of a number of secretory glands and of both smooth and striated muscle (Dale, *et al*, 1936). Acetylcholine (Ach) is a small molecule with a simple structure. It has a positively charged nitrogen atom with four attached methyl groups. Thus Ach is classified as a quaternary amine

Acetylcholine is synthesised in a single reaction from the precursors *acetyl coenzyme A* (acetyl *CoA*) and *choline.* The reaction is reversible, although the equilibrium is strongly shifted in favour of acetylcholine. The synthesis is catalysed by the enzyme *choline acetyltransferase* (ChAT). The by-product generated by the reaction is free coenzyme A (Feldman *et al*, 1997).

ChAT is found primarily in isolated nerve terminal preparations (synaptosomes) and also is thought to exist freely in the nerve terminal cytoplasm. CoA is found within mitochondria and contains the purine nucleotide adenine, the vitamin pantothenic acid and other chemical groups. The acetyl group forms an attachment with the

sulfhydryl group at the bottom of the hydrocarbon chain to form acetyl CoA. This is synthesised mainly by the pyruvate dehydrogenase complex, located in the mitochondrial matrix of nearly all cells. Choline is commonly found in foods such as vegetables, egg yolk, kidneys, liver, seeds and legumes. It is also produced in the liver. Choline enters the blood stream and is carried across the blood-brain barrier by a specific carrier system located in the membrane of capillary endothelial cells (Partridge, 1984).

The rate limiting process in acetylcholine synthesis appears to be choline transport, the activity of which is regulated according to the rate of acetylcholine release, which is stored in synaptic vesicles. Release occurs by exocytosis in response to calcium entry resulting from depolarisation of the nerve terminal following the arrival of an action potential. Acetylcholine then diffuses across the synaptic cleft to act upon acetylcholine receptors (Feldman *et al*, 1997).

Early studies (Dale, 1914) showed that peripherally administered acetylcholine effects were mimicked by muscarine, an alkaloid found in a mushroom (*Amanita muscaria*), whereas other effects were mimicked by nicotine, an alkaloid from a tobacco plant (*Nicotiana tabacum*). These findings led to the classification of acetylcholine receptors into nicotinic and muscarinic subtypes. Nicotinic actions are always excitatory and occur very rapidly. The receptors belong to the superfamily of ligand gated ion channels. The muscarinic actions can either be excitatory or inhibitory. They have a longer latency of onset. Muscarinic receptors belong to the superfamily of G protein coupled receptors. Multiple subtypes have been discovered for both the nicotinic and muscarinic receptors. In the periphery nicotinic receptors mediate ganglionic and skeletal muscle responses, and muscarinic receptors mediate cardiac and smooth muscle responses. These receptor types are also found in the central nervous system of which the majority of the responses are reported to be muscarinic and their activation is associated with a variety of actions (Feldman *et al*, 1997). For example, muscarinic receptors appear to mediate the main behavioural effects associated with Ach, namely the effects on arousal, and on learning and short-term memory.

Inactivation

The principal mechanism for terminating the synaptic action of acetylcholine involves the hydrolytic breakdown of acetylcholine. Enzymes responsible for catalysing this reaction are generally known as cholinesterases because they cleave the ester linkage between the choline and acetate moieties of the acetylcholine molecule. There are two distinct types of cholinesterases: *acetylcholinesterase* (AChE) and *butyrylcholinesterase* (BuChE). The two enzymes are encoded by separate genes and differ in their substrate specificity and tissue distribution.

AChE is a glycoprotein made up of one or more subunits. The various forms of AChE can be subdivided into heteromeric and homomeric families as well as into globular and asymmetric forms. This diversity is produced by alternative mRNA splicing. Asymmetric forms of AChE are so named because they possess a tail made up of three collagen filaments wound in a helical arrangement. This form is found mainly at the neuromuscular junction, where it is secreted by muscle and nerve cells. The enzyme molecules then become attached to the extracellular matrix by their collagenous tails (Review; Massoulie, *et al* 1993)

The globular forms are more complex due to their heterogeneity. For example G_1 and G_2 each have two variants, a hydrophilic form found in the cytoplasm and a glycophospholipid-linked form that is anchored to the cell membrane. G_4 also exists in two forms, a homomeric hydrophilic form and a heteromeric form with a lipid membrane anchor (Taylor and Radic, 1994). It is likely that in the brain, acetylcholine released into the synaptic cleft is degraded mainly by the membrane bound forms of G_1, G_2 and G_4.

Table1: The various molecular species of Acetylcholinesterase (A: asymmetric, G: globular)

HETEROMERIC	**HOMOMERIC**
Lipid Linked (G_4)	G_1 (Exon 4)
Asymmetric (A_{12})	G_1
	G_2
	G_2 - glycophospholipid linked ($G_{1,2}$)
	G_4 - hydrophilic ($G_{1,2,4}$)

The active domain of AChE comprises two subsites critical for its functioning. One of these sites is termed the anionic subsite, which has an important role in binding part of the substrate to the enzyme molecule. The other region is the active site, termed the esteratic site, which possesses a serine residue that is responsible for the hydrolytic action. Following binding of acetylcholine to the active site of AChE the ester bond of the acetylcholine molecule is broken and choline is released into the surrounding medium, where it is taken up by the presynaptic nerve terminal. The acetate group then becomes bound to the serine residue resulting in an acetylated enzyme. A molecule of water then rapidly reacts with this unstable intermediate to liberate the acetate group and regenerate the active enzyme (Feldman *et al*, 1997).

Distribution

The location of cholinergic cell groups and pathways in the brain has been accomplished using markers associated with cholinergic neurones, the most important of which uses staining procedures for ChAT which can be labelled by inmmunofluorescence. Acetylcholine itself cannot be made visible by histochemical techniques. The localisation of acetylcholinesterase, which can be readily stained, is sometimes used to indicate the presence of acetylcholine. However, its distribution is widespread and not specific to cholinergic pathways. The distribution of central cholinergic pathways has been illustrated in figure 1.

Cholinergic neurones that play an important role in the functioning of the extrapyramidal motor system can be found in parts of the striatal complex, including the caudate putamen, nucleus accumbens and the olfactory tubercule. They serve as interneurones and can receive glutamatergic afferents from the neocortex and dopaminergic input from the substantia nigra.

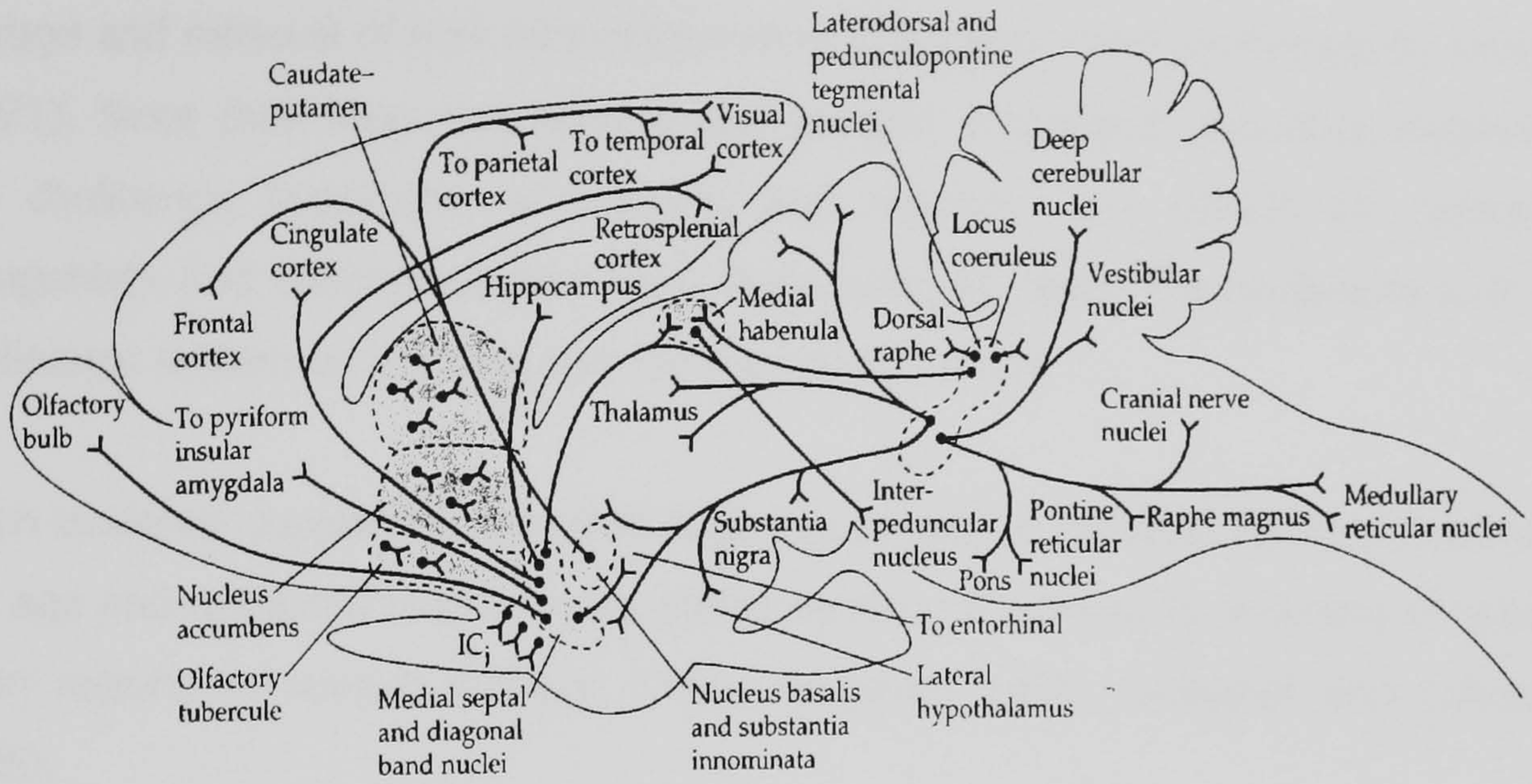


Figure 2: Distribution of cholinergic cell groups and projections in the rat brain (Woolfe, 1991).

The basal forebrain contains a major cholinergic projection system. The main rostral cholinergic cell groups of the basal forebrain are located in the medial septal nucleus and the vertical limb nucleus of the diagonal band of Broca. These send projections to the hippocampus and the limbic system. The caudal portion of this system is represented by cells in the nucleus basalis, the substantia innominata and the nucleus ansa reticularis. These neurones innervate all parts of the neocortex. The basal forebrain cholinergic neurones appear to play an important role in memory and other cognitive functions (Feldman *et al*, 1997; for full review: Woolfe, 1991).

The hippocampus is a brain structure, known for its role in cognitive functions such as memory, learning and spatial orientation (O'Keefe *et al*, 1978; Olton *et al*, 1976, Eichenbaum, 1992). A neurotransmitter system vital to the physiological functioning of the hippocampus is the cholinergic projection of the medial septum-diagonal band complex. The view that the septohippocampal cholinergic system is involved in regulating memory is supported by findings that memory performance is correlated with cholinergic activity in the hippocampus, as measured by ChAT activity, Ach level and high affinity choline uptake (Decker, 1991).

Learning and memory

The first experimental studies, in which the role of cholinergic synapses in the storage and retrieval of new information were evaluated, were performed by Deutsch (1971). Since then there has been a vast amount of research providing support for the cholinergic hypothesis of learning and memory. The effects of cholinergic antagonists and cholinomimetics have been used to study the involvement of the cholinergic system on learning and memory performance.

Much evidence supporting the cholinergic hypothesis is provided by reproduction of the age and dementia related impairments by drug treatment or lesioning of specific brain regions in animals (Fibiger, 1991; Andrews, 1994; Gallagher and Colombo, 1995).

Cholinergic dysfunctions induced either by lesions of cholinergic nuclei or by transections of major cholinergic pathways, have been shown to induce specific disturbances in memory and learning performance. Deficits in learning and memory can be achieved with cholinergic lesions of the hippocampus (Hagan, *et al*, 1987; van der Staay *et al*, 1989). This can also be achieved by transections of the fimbria fornix, lesions of the vertical limb of the diagonal band of Broca or the medial septum (Mesulam *et al*, 1983). Lesions of the nucleus basalis magnocellularis (nbm) lead to a decrease in cortical ChAT and a decline in performance in learning and memory tasks (Smith, 1988).

Blockade of muscarinic receptors by atropine or scopolamine has been shown to interfere with the acquisition and maintenance of many different kinds of learning tasks (Fibiger, 1991; Hagen and Morris, 1987; Spencer and Lal, 1983). Scopolamine induced memory impairments have gained widespread acceptance as a model of dementia (Broks et al, 1988). In animal studies, for example scopolamine disturbs spatial orientation in water and radial maze tasks (Blokland et al, 1992). In addition, acetylcholinesterase inhibitors, such as physostigmine, which enhance the availability of Ach in the synaptic cleft, were able to reverse the scopolamine-induced deficit, indicating that the cognitive deficit is cholinergic in nature.

1.3 Cognition enhancers

A cognition enhancer is a compound that improves cognitive function, such as learning, consolidation and/or retrieval (Wenk and Olton, 1989), i.e. that increases information processing capacity. A cognition enhancer should not have other classical psychopharmacological activity (Gamzu, 1989), with little or no side effects (Porchel, 1988) and have a very low toxicity. It should also readily cross the blood brain barrier.

Maitre and Pepeu (1989) have stated that the goal of a cognition enhancer is "*not to improve the facilities of learning and memory but rather to find remedies for their impairment in the aged and, as a corollary to that, the means of retarding the deteriation of these faculties*"

Since there is at present no cure for AD, current treatments are palliative in nature. Most treatment strategies have been based on the cholinergic hypothesis of cognitive dysfunction in AD (Bartus *et al,* 1982). To counteract the cholinergic decline in the central nervous system three possible strategies have been described (Drachman et al, 1982)

- Initially, it has been proposed that large supplements of the Ach precursor choline or of lecithin (phosphatidylcholine) might be able to increase the availability of Ach in the brain. However this therapeutic approach was unsuccessful. A severe limitation to this strategy was that only 1% of plasma choline is incorporated into Ach, the remainder enters alternative metabolic pathways (McGeer, 1984; Mohs and Davies, 1985).

- Agonists that can be directly administered to stimulate post-synaptic cholinergic receptors, such as nicotine and arecoline (Flood and Cherkin, 1988). These have disadvantages due to their short duration of action narrow therapeutic window and the lack of selectivity.

- Cholinesterase inhibitors, which inhibit the enzyme responsible for the cleavage of acetylcholine are a second possible treatment in Alzheimer's disease. By

preventing the breakdown of acetylcholine in the synaptic cleft, it thus prolongs the action of this neurotransmitter.

Tacrine (Cognex), [tetrahydroaminoacridine (THA)] is a potent, centrally acting first generation cholinesterase inhibitor which acts reversibly (Dawson, 1990) and non-competitively (Patockat, 1976). This compound has been shown to improve cognitive performance in Alzheimer patients (Summers *et al*, 1989; Gamzu, 1990) and also in animal studies. In animal studies tacrine has shown no effects in young intact rats in the spatial reference memory version (stable platform position) of the Morris water escape task or spatial working memory version (reversal of escape platform). However it has been shown to alleviate the cognitive deficits produced by medial septal lesions and age related impairments using the reference memory paradigm (Riekkinen *et al*, 1991) and also to alleviate scopolamine-induced amnesia in the rat (Jackson, 1996).

In addition to the cholinesterase inhibitory action and its effects on nicotinic and muscarinic acetylcholine receptors, tacrine possess a very complex pharmacology and has been reported to affect other neurotransmitter systems including noradrenaline, dopamine, glutamate and 5-hydroxytryptamine (Freeman and Dawson, 1991). Tacrine induces a high incidence of peripheral cholinergic toxicity and has low oral bioavailability. Overall, with the compound's lack of specificity, transience of effects and concerns over the risk/benefit ratio indicated that more specific compounds were required.

Metrifonate: Background Information

Metrifonate (0,0-dimethyl- (1-hydroxy-2, 2,2-trichloroethyl)-phosphonate) is an organophosphorus compound and is a member of the class of second-generation cholinesterase inhibitors (Giacobini, 1991). These are characterised by high penetration through the blood brain barrier, long lasting and selective inhibition of cholinesterase with few cholinergic side effects. In a study with Alzheimer disease patients, metrifonate was found to be effective in treating decline (Becker *et al*, 1990). The compound was taken through into Phase III clinical trials as an Alzheimer therapeutic agent (Gelina *et al*, 2000). Methods of testing cognitive function in

elderly and demented patients are augmented by batteries of computerised tests (Robbins et al, 1989). The Cambridge Automated Neuropsychological Test Battery (CANTAB) was developed to adapt paradigms for testing animal models of dementia in order to relate the findings to human patients. The tests of pattern and spatial serial recognition memory and delayed matching to sample process analogues are included.

Metrifonate is not a cholinesterase inhibitor by itself. In aqueous alkaline solutions it is spontaneously dehydrochlorinated non-enzymatically to the organophosphate ester dichlorvos (2,2-dichlorovinyl dimethyl phosphate). It is thought that metrifonate acts as a prodrug via non-enzymatic release of dichlorvos, which covalently phosphorylates the acetylcholinesterase (Hinz *et al*, 1996). In human blood *in vitro*, the half-life of metrifonate is about 60 minutes compared to 10 minutes for dichlorvos (Villen *et al.*, 1990). Therefore, it is not possible to accumulate dichlorvos after *in vivo* administration of the parent drug, metrifonate. Due to this slow release formulation, metrifonate has fewer side effects than many other cholinesterase inhibitors. Dichlorvos is 100 times more potent than metrifonate (Hinz, *et al*, 1996).

In a number of studies metrifonate has been shown to improve the cognitive performance of animals in various behavioural models. Using the passive avoidance paradigm with rats, metrifonate was found to alleviate scopolamine- induced or basal forebrain lesion induced deficits in rats (Itoh, *et al*, 1997). Riekkinen *et al* (1996) described a beneficial effect of metrifonate on passive avoidance performance deficits induced by scopolamine or by medial septal lesions. Metrifonate facilitated passive avoidance retention in 23-month-old and 27-month-old rats (Riekkinen, *et al*, 1996). The effects of metrifonate on cognitive performance have also been investigated in the Morris water escape task, in which the rats are trained to locate an invisible submerged platform in a water pool. The compound facilitated acquisition of the task by young (van der Staay *et al*, 1996b), elderly [19- month old (Blokland *et al*, 1995)] and aged (23- and 25, but not 27-month old) rats (Riekkinen *et al*, 1996; van der Staay *et al*, 1996b). Metrifonate treated rats needed less time and swam shorter distances to find the escape platform than did age-matched controls.

Cognitive performance after sub chronic administration of metrifonate has also been investigated in aged rabbits (33- to 34- months old) in a classically conditioned eye-blink task. Ageing rabbits and humans show an impaired performance in this task (Disterhoft *et al*, 1999). The animals were pre-treated for 1 week with Metrifonate at one daily dose. The animals were then trained on classical eye-blink conditioning, with drug administration continuing. Associative learning was improved by metrifonate compared with the aged control group (Kronforst-Collins *et al,* 1997).

Side effects and Administration schedules

Side effects, induced by metrifonate treatment are mild in comparison to those of other cholinesterase inhibitors (Hallak and Giacobini, 1989), follow maximum cholinesterase inhibition and disappear shortly after administration (Dubois and Cotter, 1955). The acute toxicity of metrifonate in animals, expressed in terms of lethality, is quite low compared to many organophosphorous compounds (for a review see Holmstedt et al, 1978). The oral LD_{50} values vary from species to species. In rats the value ranges from 400-700mg/kg (Jones et al, 1968). Acute high doses of metrifonate induce adverse symptoms such as salivation, tremor and diarrhoea, which are typical for cholinergic drugs (DuBois and cotter, 1955; Edson and Noakes, 1960)

After acute administration of metrifonate to rats (80 mg/kg im; 125 mg/kg p.o), ChE activity in brain and blood recovers within 24 hours (DuBois and cotter, 1955; Hallak and Giacobini, 1989). Repeated administration of metrifonate leads to a long lasting inhibition of blood ChE (DuBois and cotter, 1955). Neurotoxicological studies showed that after 13 weeks of metrifonate treatment (Chan and Peters), no toxicological damage were observed on rat brain, spinal cord, sciatic nerve and other organs at doses of up to 5000ppm metrifonate.

In this dissertation the term "*subchronic*" is used for repeated drug administration, lasting up to 4 weeks. The term "*chronic*" is used for drug treatment lasting longer than one month.

1.4 Animal studies of behavioural dysfunction

The search for valid animal models capable of mimicking human pathophysiology and which enable us to detect and optimise therapeutically interesting drugs is essential in pharmacology. Despite some controversy about the validity of extrapolating data from animal models to human physiology their use is necessary before carrying out clinical trials in humans. However, no single animal model of Alzheimer's disease, which combines all the relevant pathophysiological and behavioural aspects of the disease, is known. Most models attempt to reproduce certain features of the disease, for example; behavioural deficits, histological stigmata or metabolic disturbances.

The most relevant information can be derived from the study of humans, however the neurobiological variables associated with behavioural dysfunctions cannot be sufficiently controlled in experimental and clinical studies with human subjects. Therefore animal models are used to answer questions about behavioural dysfunctions and their underlying neural substrates. Animal models have been defined as follows: " *An animal model in the behavioural neurosciences is a living organism used to study brain-behaviour relationships under controlled conditions, with the final goal of gaining insight into these relationships in humans and/or (a) species other than the one(s) studied, or in the same species under conditions different from those in which the study was performed*" (van der Staay, 1998).

Validity

Criteria have been proposed for the validity of animal models of behavioural dysfunctions, such as those seen in dementia. Validity is a central concept in science and it centres on the question as to how close is the model to reality and how relevant results are for to humans.

Animal models should possess face validity, predictive validity and construct validity (D'Mello & Steckler, 1996). These different forms of validity help us to identify weaknesses or defects in a particular model.

- Face validity evaluates the degree of similarity between measures in the animal model and their correspondence in humans. i.e. an animal model should meet the requirement that it resembles the condition to be modelled with respect to its aetiology symptomology, underlying processes and treatment (McKinney & Bunney, 1969).
- Predictive validity indicates how well the animal data predicts behaviour in the situation it is supposed to model. i.e. if it allows extrapolation of the effect of a particular experimental manipulation in one species to other species, including humans.
- Construct validity refers to the theoretical clarification of what a test measures, establishing relationships that are based on definition of a trait, such as intelligence or emotional reactivity.

In general, face validity is the naive level, i.e. the test appears valid because of a perceived resemblance between the model and the situation or process to be modelled. Predictive validity is the empirical level, i.e. data shows that the outcome of the model has some predictive value and construct validity is the theoretical level.

Type of drug screening models

Models have provided information about potential biochemical and structural changes that might underlie associative learning. Animal models that reproduce brain damage that impacts upon cognitive function have indicated the relative importance of various brain structures for aspects of cognitive functioning. These models, primarily developed for basic research purposes, can be used to identify cognitive enhancers, i.e. drugs for the treatment of cognitive deficits associated with AD.

Normal subjects

Normal subjects are used most widely to assess the effects of putative cognition-enhancing compounds. Though they may not fulfil any criteria concerning the neuropathology or the occurrence of behavioural dysfunctions and consequently, in a strict sense cannot be considered an animal model, there is an underlying assumption that compounds that improve performance in normal animals would also be useful for the treatment of patients (Gamzu, 1985).

Old subjects

The use of aged animals represents a naturally occurring impairment model. The model is based on the similarity between behavioural symptoms in old rats and in patients suffering from AD, such as sensorimotor dysfunctions (Markowka *et al*, 1990), decreased social activity (Spuijt, 1991) and cognitive impairments. The advantages of this model are that small rodents have a relatively short life span of 2-3 years, their environment can be controlled and they show age- related impairments (Elias, 1976). However, these models do not mimic the specific neuropathological changes underlying AD.

Genetic Lines

If a selected population is inbred for several generations, a genetic line of biologically uniform animals is obtained. For example, genetic lines can be developed which have deficits in cognitive functioning. Closely related to the model of the aged animal is a mouse model genetically selected to show characteristics of accelerated ageing. The senescent–accelerated prone mouse shows an earlier than normal onset of age- related deterioration in learning and memory (Miyamoto *et al*, 1986).

Selective impairment- Pharmacological Manipulations

Several lines of evidence support the notion that a loss of cholinergic function contributes to the cognitive deficits seen in AD (Bierer *et al*, 1995). Therefore pharmacological disruption of cholinergic function has been an approach in modelling cognitive deficits in AD. Scopolamine, a muscarinic acetylcholine receptor antagonist is used to produce cognitive deficits originally described as resembling those normally occurring in aged subjects (Drachman, 1977). It is perhaps the most widely used model of memory impairment of AD. The impairment induced by scopolamine is pervasive and can be indexed by performance on such tasks as object discrimination learning (Ridely et al, 1984), radial maze performance (Okaichi et al, 1989), and acquisition and retention in the Morris water maze (Dunnett et al, 1991) and can impair passive avoidance (Matsuoka et al, 1992). Scopolamine is also thought to disrupt attentional functions. Several studies have provided experimental evidence that scopolamine affects the allocation of attentional processes rather than impairs memory per se in humans (Dunne 1986; Warburton et al, 1992)

Although the disruptive effects of scopolamine, on a variety of cognitive tests, are robust and reproducible it has a few problems and may not be ideal to model the mnemonic deficits in early stages of AD. The effects of scopolamine are reversible whereas AD is a progressive, irreversible disease. Scopolamine reduces cholinergic activity post-synaptically, whereas reduced muscarinic neurotransmission in AD is mainly pre-synaptic. The nature of the disruptive effects is often only partially known and test compounds aimed at reversing the deficit could change the metabolism or tissue disposition of the cholinergic antagonist (Decker, 1995).

CNS Specific Lesions

Lesion models have been extensively used to test compounds for cognition enhancement, in an attempt to mimic the neuroanatomical, histological and neurochemical correlates, which are observed in post mortem samples of AD affected brain. Lesions focus primarily on the cholinergic innervation areas of the basal forebrain and on higher regions such as the cortex and hippocampus, which show pronounced deterioration in AD (Bierer *et al*, 1995).

Transgenic Models

Transgenic approaches allow the development of animal models of human disease. Whenever a disease is shown to be caused or influenced by specific, identified human genes, introduction or modification of those genes in animals can mimic the geneticity of the human pathology. As discussed previously, genes that are involved in AD have been identified. Mutations in the genes presenilin-1 (PS-1) and presenilin-2 (PS-2), and, additionally mutations in the human amyloid precursor protein (APP) gene, cause changes in the processing of APP, leading to amyloid formation in the brain (Nitsch, 1996).

The key pathological changes of AD that transgenic rodents would express is the senile plaques and neurofibrillary tangles which could be produced by introducing human transgenes carrying one or more disease causing gene mutations (Loring *et al*, 1996). Two genetically engineered AD models are based on transgenic mice expressing familial AD mutations or fragments of normal APP: the Minnesota mouse (Hsiao *et al*, 1997) and the Exemplar/Athena mouse (Games *et al*, 1995). The APP mutations they express cause an early onset of familial AD in humans, possibly as a

result of the increased production of total ß-amyloid and ß-amyloid$_{1-42}$. Both models are characterised by the accumulation and deposition of amyloid.

Selection of Animal Species.

The use of non-human primates in science does not seem to be wholly necessary in this field. Non-human primates do not offer many advantages compared to other animals. They are difficult to handle, and cannot be obtained in sufficient number. More important, it is impossible to obtain genetic homogeneity and they are impractical for conducting longitudinal studies. Moreover, their close evolutionary relationship to humans does guarantee neither similar pharmacodynamic and pharmacokinetic responses, nor behavioural homologies (D'Mello & Steckler, 1996). Small animals such as rodents and pigeons are preferentially used. They are easy to handle, have a relative short life span and can be studied in controlled conditions. Rodents are particularly well suited for cognition and ageing research, which is comparable to that of humans (Steckler & Muir, 1996). In addition, dissociation between chronological and biological ageing occurs in rodents (Collier & Coleman, 1991).

Animal studies can thus be of great help in determining the relative contribution of various factors regarding ageing in learning and memory, which will indirectly foster dementia/cognitive research. Some rat or mouse strains are more suited to certain tests than others. Differences between strains must first be identified to be able to evaluate their influence on cognitive performance and drug effects. Strains may react differently in behavioural tasks and, as a result, performance may be confounded by different factors e.g. changes in attention compared to changes in motivation. Furthermore, small differences in feeding behaviour and food requirement of different strains can have fundamental effects on performance. In addition, strains certainly differ in their pharmacodynamic and pharmacokinetic characteristics, which in turn may affect drug evaluation. Strain, but also age and gender are important factors that need to be considered for evaluating cognitive performance in rodents.

Ethical Considerations

Man's utilization of animals for any purpose is a privilege that carries with it a series of responsibilities. In the case of research animals, these fall into three broad categories: 1) technical, 2) ethical, and 3) legal. The technical include factors such as selecting the proper animal, providing the proper environment and care, and designing experiments to use as few animals as possible while maintaining scientific validity. Ethical considerations stem from an inherent respect and reverence for life which anyone utilization animals should have. This carries with it a responsibility to assure proper care and humane treatment. Some legal requirements have resulted from the ethical considerations. While these vary considerably, nearly all countries have some laws related to this subject.

Selection of the task

The first requirement of any test of animal cognition is that it really measures cognition, which is defined as the 'faculty of knowing, perceiving, (and) conceiving as opposed to emotion and volition', rather than a non-cognitive process, for example sensory, motoric and non-specific arousal processes. The tasks should be chosen for their specificity and selectivity toward the behavioural phenomena observed in the disease. Short-term memory and spatio-temporal abilities seem to be first affected in AD (Adams, 1997). Tests have therefore been developed that focus on these processes in which animals have to develop and learn new strategies, to memorise and retrieve cues in order to solve the task successfully.

Pre-clinical evaluation of drugs affecting memory in animals involves observing their behaviour in experiments consisting of a presentation of information (the training stage) followed by a delay and the opportunity for performance (the test stage). Higher cognitive processes can be evaluated by providing situations in which reorganisation of the information presented is necessary before the appropriate response is made. A task selected for drug evaluation should be *replicable* by an independent group of researchers; as stated by van der Staay (1998) "*results are preliminary as long as they have not been corroborated, and preferably by investigators other than those who originally performed the investigation*". The cognitive measures are *specifically* affected by selected drugs in a *sensitive* manner

and should be a part of a *battery* of cognitive tasks. It is also necessary to remember that factors such as housing conditions, biological rhythm, stress and route of drug administration can affect test data significantly as can strain, age and gender (Andrews, 1996)

One way to improve cognitive research is to refine existing animal models and to create new ones. An animal model should initially be derived from a theory, which will then be confronted. If the theory is correct, the model already possesses construct validity and then needs to be transformed to fulfil further criteria. At this point, predictive and face validity should be increased. Face validity may be absent or only partially present because of species-specific particularities. Once a cognitive enhancer is identified, particularities of the drug can be investigated to develop quick tests.

1.5: Tests to assess behaviour in animals with Cholinesterase Inhibition

In this thesis a number of behavioural tests are used to determine the effects of cholinesterase inhibitors at the cognitive level.

Passive Avoidance Task

The passive avoidance task is based on an instinctual effect in which, when you hurt yourself unexpectedly, e.g. have a mild electric shock you will avoid or refrain the action that caused you the discomfort. Although avoidance tasks are seldom, if ever used with patients they are widely used in animal research and because they are tests of learning and memory, results are often used to model Alzheimer's disease. The test is considered as a useful early screening test to identify putative cognition-enhancing compounds in rodents and has been extensively used in the majority of drug screening programs (Iversen, 1997) mainly due to being fast and cheap. Many patents are based on positive effects of a compound in this task. Compounds are usually tested in scopolamine- treated rats.

Standard Morris water escape task

The standard Morris water maze (MWM) was developed by Morris (1984) as a device to investigate spatial learning and memory. Age related deficits in spatial maze learning are evident in both place and route learning in a number of mammalian species (Barnes, 1979; Ingram, 1988; Mclay et al, 1999). In this task, a rat is trained to localise a submerged platform and measures predominantly spatial reference memory (Mundy *et al*, 1990). Reference memory holds trial- independent information (Barnes, 1990).

Many studies have examined the role of specific brain regions that could be involved in Morris water maze learning. The involvement of brain regions in spatial navigation is complex and may comprise numerous regions (Cain et al, 1996). It has been well established that the hippocampal formation is essential for spatial learning (O'Keefe and Nadel, 1978; Barnes, 1988). Lesion studies have shown that damage to the hippocampus and virtually all its associated structures (fornix, septum, entorhinal cortex, subiculum, post subiculum) induces severe and permanent deficits in a wide variety of spatial abilities (Rasmussen et al, 1989). Other regions include the striatum (where bilateral lesions have shown to affect Morris water maze performance; Whishaw et al, 1987), the basal forebrain (Brandeis et al, 1989), cerebellum (Lalonde, 1994; Leggio et al, 1999) and the neocortex (Brandeis, 1989).

The Morris water task has been used in studies on the involvement of neurochemical systems in place learning and memory and the effects of neuropharmacological manipulation on spatial functions (Brandeis, 1989; McNamara et al, 1993). McNamara and Skelton (1993) reviewed the involvement of different neurotransmitter and modulator systems in spatial learning, and suggested that only the cholinergic, glutaminergic and some peptidergic systems may really be involved in learning, whereas GABA, opioids or biogenic amines are either detrimental or unrelated.

There have been a few studies that have examined age-related vulnerability in route learning or topographical memory in humans. Elderly individuals have deficits in navigation and develop behavioural patterns to avoid unfamiliar routes and places

(Burns, 1999). Impairments in navigation are often apparent in the early stages of dementia (Klein et al, 1999; Passini et al, 1995).

The two choice water escape task

In a variation of the standard Morris maze task, as discussed above, the subjects are given trials which consisted of two runs: an information run and a test run which is separated by a retention interval. The information run consists of a forced choice trial during which subjects are forced to the correct choice section by closing of the incorrect sections. The test run consists of a free choice trial, during which the doors to all choice sections are open.

The two choice water escape task is a water version of a delayed matching to position or sample task that can be either visual or spatial and can be performed in either operant conditioning chambers or mazes. Although there are various various they generally involve the presentation of a conditional (sample, choice) stimulus followed by a delay and then two or more discriminative (comparison) stimuli. Only one of the comparison stimuli designates a correct choice and is given a reinforcer on any given trial. Patients with early stages of AD show increased impairment, relative to controls on visual delayed matching to position task (Money et al, 1992; Sahakian et al, 1988).

The task has many advantages of the Morris water maze, in that it involves no food or water deprivation which could interact with pharmacological or neural manipulations and ultimately alter behaviour that indirectly influence cognitive performance scores. It appears to be less aversive than shock motivated tasks. The task provides a choice measure of performance as well as a latency measure. Choice is a better measure of cognitive processes than is response latency because choice is influenced less by variables that alter motor ability and activity levels. The task has proven to be sensitive to age (Means and Kennard, 1991), to oestrogen therapy in ovariectomised female rats (O'Neal *et al*, 1990) gender (Means and Dent, 1991) and to Piracetam and BMY 21502, both of which are putative cognition enhancers.

Timing Behaviour

Timing behaviour is thought to be a cognitive function of the brain in terms of a subjects' ability to control the way in which stimulus information is integrated over time. Neuropharmacological studies have shown some of the neural and behavioural mechanisms involved in timing and time perception. The 'internal' clock is used for measuring the speed or durations in which information is integrated and this appears to be linked to dopamine function in the basal ganglia and the memory storage process used for the representation of the duration's of prior events appears to be linked to acetylcholine function in the frontal cortex. These two systems appear to be linked by frontal striatal loops (Meck, 1996). Past studies have shown that distortions in perceptions of time accompany a number of different neurological disorders (Fraisse, 1984; Goody, 1969; Malapani et al, 1993; Meck, 1983; Nichelli et al, 1993) Degenerative diseases of the basal ganglia can cause subjects to underestimate time in the short range (O'Boyle et al, 1996; Freeman et al, 1996) while causing them to overestimate time in the longer time ranges from minutes to second ranges. Studies have shown that patients with AD have defective accuracy and precision in time estimation (Carrasco et al, 2000; Nichelli et al, 1993).

In this study the peak interval procedure is used which is an operant conditioning schedule in which a lever is presented and the animal is free to respond at any time, but only the first response after a fixed duration (e.g. 20 seconds) is reinforced. In the peak interval (PI) procedure some trials are identical to the FI procedure where others consist of the lever presented for a time that goes beyond the fixed duration (e.g. 50 seconds), and no reinforcement is given.

1.6: Aims of Study

Alzheimer's disease is accompanied by pronounced neurodegenerative changes in the brain (Braak and Braak, 1991). These changes are likely to lead to impairments in cognitive performance, such as attention, learning, and memory, depending on the quality and extent of the damage. Pharmacological therapy might ameliorate cognitive functioning in affected patients. One such therapy is metrifonate, a second generation cholinesterase inhibitor (Giacobini, 1991) that has been show to be effective in patients with mild to moderate AD (Gelina *et al*, 2000) and in various behavioural tasks (Riekkinen, *et al*, 1996; van der Staay *et al*, 1996b; Schmidt *et al*, 1997; Itoh, *et al*, 1997; Disterhoft *et al*, 1999).

Firstly the aim of this thesis was to look further in to the biochemistry of the reference compound, metrifonate. Specifically to look at the process of how the compound induces tolerance to cholinergic side effects after subchronic administration.

The main aim of this study was to confirm the behavioural effects of metrifonate, serving as reference cognition enhancers in known behavioural models in the rat to determine that the results are reproducible across laboratories and animals/experiments and then to determine these effects in a second species i.e. the mouse. It was also planned to assess new methods, using metrifonate, in order to evaluate their potential for investigating future cognitive enhancers. Finally, the effectiveness of metrifonate to be used as a reference compound for future studies is to be discussed

Adaptation to Prolonged Administration of Metrifonate: Effects on the Periphery and CNS

Abstract

Metrifonate, a long lasting and well-tolerated cholinesterase inhibitor was administered once daily for up to twelve weeks at oral doses of 0, 10, 30, 50, 100 and 150 mg/kg. During the sub chronic treatment period, experiments were performed to determine behavioural and neurochemical changes. Cholinesterase activity was determined in both central and peripheral tissues. It was found that 50 mg/kg Metrifonate caused a 30 % reduction in the cholinesterase activity in the brain. After 12 weeks of treatment erythrocyte cholinesterase inhibition compared to the shorter period of treatment period of 6 weeks mediated the same level of about 60%. The reduction in cholinesterase activity was not reflected in peripheral tissues suggesting that inhibition of peripheral cholinesterase in the blood does not necessarily lead to peripheral inhibition and thus to peripheral side effects.

In determining the development of behavioural tolerance centrally and peripherally cholinergic compounds (bethanechol, neostigmine, nicotine, oxotremorine and donepezil) were administered to metrifonate pre-treated rats. No changes were seen with the peripherally acting muscarinc agonist, bethanechol or the cholinesterase inhibitor, neostigmine in adverse events or body temperature. In response to the centrally acting muscarinc agonist oxotremorine, a subsensitivity was observed in only one of the side effects i.e. clonic seizures whereas nicotine did not induce any changes. These two compounds also produced no changes in sensitivity in body temperature. Donepezil, a centrally acting cholinesterase inhibitor produced a supersensitivity in both adverse effects and to body temperature. We conclude that the development of tolerance is a centrally mediated phenomenon and is possibly caused by unknown changes to the cholinesterase enzyme within the brain.

Introduction

The cholinergic hypothesis of memory dysfunction in ageing people and Alzheimer's patients (Bartus *et al*, 1982; Coyle *et al*., 1993) led to the development of compounds designed to increase the deficient acetylcholine levels in the brains of these patients. These compounds, known as second generation cholinesterase inhibitors are long lasting, safe and well tolerated. They aim to restore levels of endogenous acetylcholine in the synaptic cleft by preventing its enzymatic breakdown by acetylcholinesterase.

Metrifonate, an organophosphorous compound acts as a pro-drug for an active metabolite (Hinz *et al*, 1996a). The slow release of the active metabolite from the parent drug ensures a smooth onset of cholinesterase inhibition. In a wide range of models of learning and memory, metrifonate has shown cognition enhancing activity (Schmidt *et al*, 1997) and data from clinical studies confirmed that this is also true for Alzheimer patients (Becker, 1990, Cummings *et al*, 1998)

Cholinesterase inhibitor treatment produces an accumulation of acetylcholine at the muscarinic and nicotinic cholinergic receptors. In high doses, this produces classical cholinergic side effects such as salivation, diarrhoea, tremor etc. However, multiple doses of some cholinesterase inhibitors induce the development of tolerance, which is shown by a marked recovery from the signs of toxicity associated with cholinergic overstimulation (Chippendale *et al*, 1972; Overstreet *et al*, 1974; Costa *et al*, 1982; Russel *et al*, 1986).

Behavioural tolerance is supposedly due to the activation of secondary processes which compensate for the increase in ACh resulting from cholinesterase inhibition. Theories of tolerance development include compensatory synthesis of enzymes that metabolise or bind organophosphates (OPs) thus resulting in increased degradation or scavenging of incoming OPs. The accumulation of acetylcholine might lead to a reduced release of acetylcholine by a pre-synaptic feedback. Accumulation of acetylcholine may cause desensitisation and a rise in the threshold of the post-synaptic membrane (van Dongen et al, 1989) or receptor down regulation. The resulting subsensitivity is not restricted to the occurrence of cholinergic side effects, but may also affect the functionality of

central cholinergic pathways including those involved in attentional or cognitive processes (e.g. Bushnall *et al*, 1991; Mcdonald *et al.*, 1988; Stamper *et al.*, 1988; Wolthuis *et al*, 1990). In the case of metrifonate one behavioural aspect of cholinergic activation, i.e. adverse reaction to cholinergic overstimulation appears to become hyposensitive, whereas beneficial effects on cognitive improvement are spared or unaffected. Alternatively, it has been suggested that metrifonate acts via a second, unknown mechanism of action (van der Staay, 1996; Itoh *et al* ,1997), which is not prone to desensitisation of procognitive effects. However, the exclusively cholinergic nature of metrifonate (Blokland *et al*, 1995; Hinz *et al*, 1996) as well as the failure of metrifonate or its active metabolite to interact with more than 60 molecular receptor targets in an *in-vitro* receptor screening (Hinz *et al* 1996) suggests a differential adaption of central vs peripheral responses to cholinergic stimulation.

The aim of this study was to determine if there is a differential adaptation of central versus peripheral responses to cholinergic stimulation. The first experiment was designed to determine whether cholinesterase inhibition is limited to central areas or if it is also a peripherally mediated phenomenon. To do this cholinesterase activity is studied in the brain and also peripheral tissues, which mediate cholinergic side effects such as salivation and diarrhoea.

In the second experiment we determined whether development of tolerance originates centrally or peripherally. This is achieved by observing behavioural side effects after introducing challenge doses of centrally or peripherally acting cholinergic compounds. The final study looked specifically at a selectively central phenomenon, that of body temperature which is regulated by ACh muscarinic receptors in the hypothalamus (Ghanta et al, 1996). Challenge doses of specific centrally mediated cholinergic compounds are administered to animals that have received sub-chronic metrifonate treatment and changes in body temperature are observed.

Bethanechol, a muscarinic agonist and neostigmine, a cholinesterase inhibitor was chosen because of their inability to cross the blood-brain barrier. Doses were chosen

that does not have greater toxic effects than metrifonate. Nicotine, oxotremorine, and donepezil, were chosen for their predominant central actions.

2.1: Comparison of Cholinesterase activity between the CNS and periphery in rats with sub-chronic metrifonate

Procedure

Animals: Thirty male HsdCpb: WU (Harlan Wistar) rats (supplied by Harlan-Winkelmann, Borchen, Germany) were used. They were approximately 10 weeks old at the beginning of the experiment. Their weights ranged from 220-250 grams. The rats were housed in pairs in standard Makrolon™ Type III cages in which food and water was continuously available, with a 12:12 hour light/ dark cycle, lights on at 07.00 am.

Treatment and processing of samples: The animals were treated daily with 10 (N = 7), 30 (N = 8) or 50 (N = 8) mg/kg of metrifonate (p.o) in an application volume of 5 ml/kg for up to 12 weeks. Control animals received vehicle only (sodium citrate buffer, pH 5.5, N=7). After 6 weeks of treatment 0.5ml of blood was collected from the tail vein. Eighteen hours after the final application the animals were killed by decapitation. Whole forebrains, salivary glands, heart, striated muscle (0.5g) from the hind leg and ileum (0.4g) were dissected over ice and homogenised in 3 volumes (w/v) of 0.9% NaCl solution. Trunk blood was collected in tubes of 0.5 ml of 0.3M EDTA, pH 7.4. The blood was then diluted (2:1) with Triton–100 followed by careful sonification.

Enzyme assay were performed on ice. 50µl of the protein solution were mixed with 10µl of 30mM aqueous solution of acetylcholine iodide containing 100,000 dpm [^{3}H] acetylcholine iodide (New England Nuclear, Boston). After 3 min of incubation, the reaction was stopped by adding 100µl of an ice-cold solution containing 1M-chloroacetic acid, 0.5M NaOH, and 2M NaCl. Finally, 4 ml of a polar scintillation cocktail (Quickscintz 501) were added to extract and quantify the formed [^{3}H] acetate by β- scintillation counting.

Analysis: The inhibition of cholinesterase activity across the selected dose range was analysed by ANOVA, with the factor Dose, complemented by post-hoc Fischer LSD comparisons. In order to analyse whether the effect on erythrocytes of chronic metrifonate changed across the 12 weeks of treatment an ANOVA with factors Dose and sample time point (6 *vs* 12 weeks) with repeated measures on the last factor was performed

Results

Cholinesterase activity in erythrocytes: (See figure 1,upper left panel)

Averaged over all groups the cholinesterase activity in the erythrocytes after 6 weeks was reduced by metrifonate treatment ($F_{3,25}$ = 5.12, $p<0.05$). Metrifonate treatment continued to affect cholinesterase activity in the blood after 12 weeks ($F_{3,25}$ = 5.21, $p<0.05$). Post hoc comparisons revealed that at both 6 and 12 weeks the 50 mg/kg dose of metrifonate differed from vehicle, producing a 40 % reduction in cholinesterase activity. Metrifonate treatment had an effect when the average across the 6th and 12th week samples was considered (GENERAL MEAN: $F_{3,25}$ = 6.33, $p<0.05$). However, no further decrease in cholinesterase activity was observed between the two sample collection points ($F_{1,25}$ = 1.35, n.s).

Cholinesterase activity in the brain (See figure 1, upper middle panel)

Metrifonate treatment caused a 30 % reduction in the cholinesterase activity in the brain ($F_{3,25}$ = 4.75, $p<0.01$) after 12 weeks of administration. Post hoc comparisons revealed that the ChE activity of the group treated with 50 mg/kg metrifonate differed from that of the vehicle treated group.

Cholinesterase activity in the heart (See figure 1, lower middle panel)

Averaged over all groups the cholinesterase activity in the erythrocytes in the heart weeks was reduced by metrifonate treatment ($F_{3,25}$ = 2.77 $p<0.05$), however post hoc analysis revealed that no dose differed from vehicle treated groups and that the differences observed was between 10 and 50 mg/kg, on the other hand was statistically confirmed.

Cholinesterase activity in skeletal muscle (figure 1, upper right panel), salivary glands (figure 1, lower left panel), and smooth muscle (figure 1, lower right panel) Metrifonate had no effects on the cholinesterase activity in skeletal muscle ($F_{3,25}$ = 0.56, n.s.), the salivary glands ($F_{3,28}$ = 0.55, n.s.), or smooth muscle ($F_{3,25}$ = 0.81, n.s.) after 12 weeks of treatment.

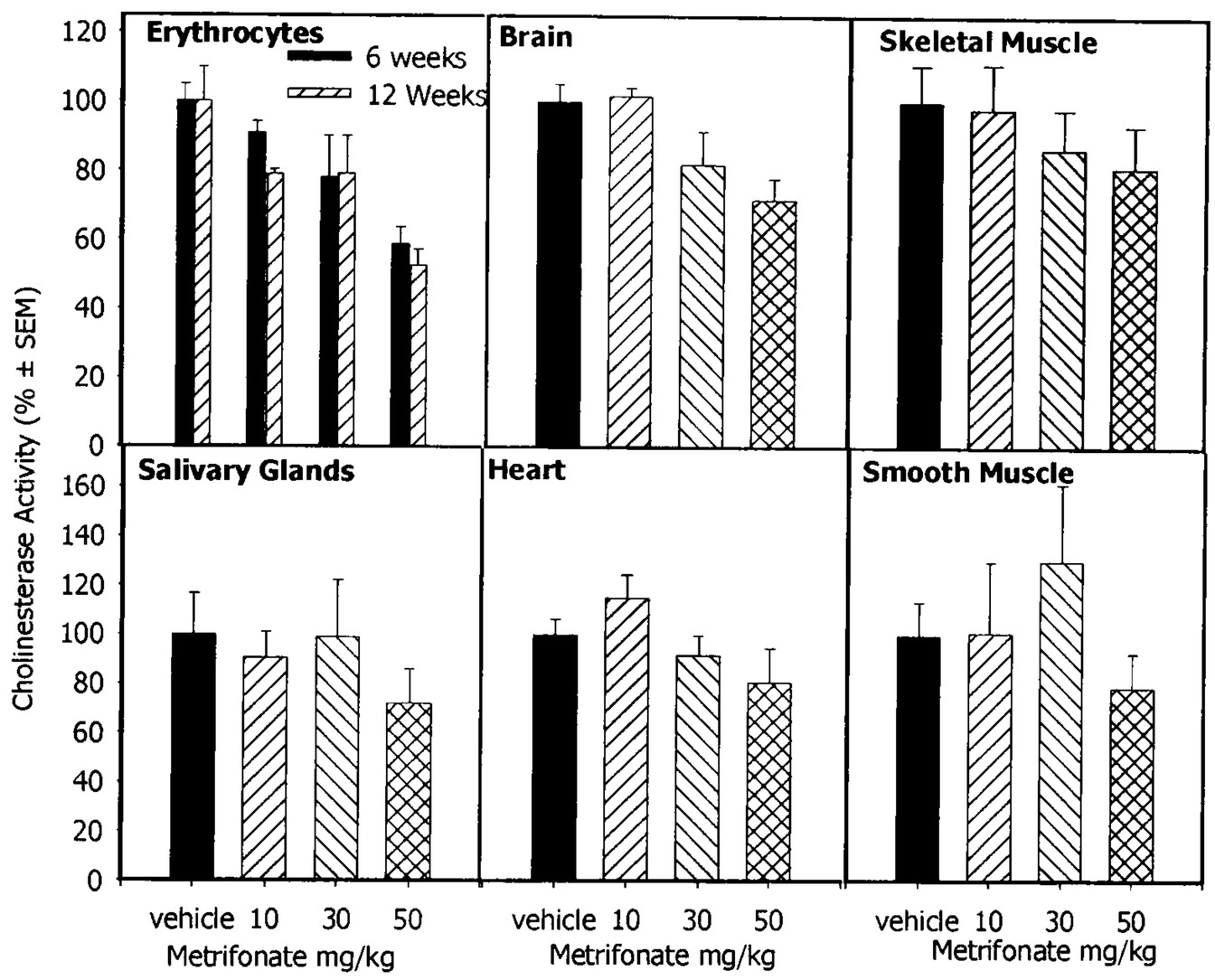


Figure 1. Effects of sub-chronic Metrifonate on cholinesterase activity in tissues in the periphery and the CNS. Data is depicted as percentage, ± SEM. N =7-8.

2.2: Study of the adverse event response to cholinergic compounds in rats with sub-chronic metrifonate

Procedure

Animals: Forty male HsdCpb: WU (Harlan Wistar) (supplied by Harlan- Winklemann, Borchen, Germany) were used. They were approximately 10 weeks old at the beginning of the experiment. Their weights ranged from 220-250 grams. The rats were housed in pairs in standard Makrolon™ Type III cages in which food and water was continuously available, with a 12:12 hour light/ dark cycle, lights on at 07.00 am.

Drug Administration: Rats (N=10) were pre-treated orally once daily with 50, 100, or 150 mg/kg Metrifonate in an application volume of 5 ml/kg for 7 weeks in order to induce steady state cholinesterase inhibition. Controls received the vehicle (sodium citrate buffer, pH 5.5) under identical conditions (see Appendix 2 for adverse event profile for sub-chronic metrifonate treatment).

Eight weeks after the start of the experiment, challenge doses of either centrally acting or peripherally acting cholinergic compounds were administered to each of the metrifonate treated groups and to the control group. The centrally acting compounds used were: nicotine, 0.7 mg/kg, oxotremorine, 0.3 mg/kg and donepezil, 3 mg/kg. Peripherally acting compounds used were: neostigmine 0.2 mg/kg and bethanechol 30mg/kg. All compounds were administered in an application volume of 2 ml/kg, i.p. simultaneously with metrifonate or vehicle. A week was introduced between each challenge dose to ensure drug washout.

Methods: Immediately after administration the animals were put singly into small Makrolon™ Type II observation cages. Their behaviour/adverse symptoms such as ptosis (drooping eyelid), salivation, sedation, diarrhea, arched back (kyphosis), exopthalmus (eye swelling), piloerection, Straub tail, tremor, clonic seizure, limb abduction, vocalisation were observed by a skilled observer every 15 min for 3 hours after administration (adverse effects observed between 0-15 minutes were also noted). The occurrence of abnormal behaviours was scored quantitatively. The animals receiving the challenge drug only served as controls for the effects of the challenge drug itself. An untreated group was not included, as previous experiments in our lab had consistently shown that vehicle treatment did not induce any observable effects.

Statistical analysis: The effects of the compounds were analysed on the sum of individual side effects over a nine-point observation period by ANOVA, with the factor Dose, complemented by post-hoc Fischer LSD comparisons.

Results

Sub-chronic metrifonate with Oxotremorine

Adverse effects were observed in all animals 3 minutes after administration of oxotremorine alone or oxotremorine with Metrifonate 50, 100 or 150 mg/kg (*see fig*

2 and table 1). Particularly evident signs were: prone position and abnormal respiration (gasping). Other symptoms observed included tremors and clonic seizures and salivation. Only clonic seizures were differentially affected by the treatment ($F_{3,35}$ = 4.33, $p<0.01$). Post hoc comparisons revealed that these seizures occurred in the group treated with oxotremorine alone, and in no other group. All other symptoms did not differ between treatment groups (Prone position: $F_{3,35}$ = 0.18, n.s.; Abnormal respiration: $F_{3,35}$ = 0.18, n.s; Head tremors: $F_{3,35}$ = 0.09, n.s; Salivation: $F_{3,35}$ = 0.67, n.s). The abnormal effects peaked around 5 minutes after administration. They were transient by disappearing at the latest about 75 min after administration.

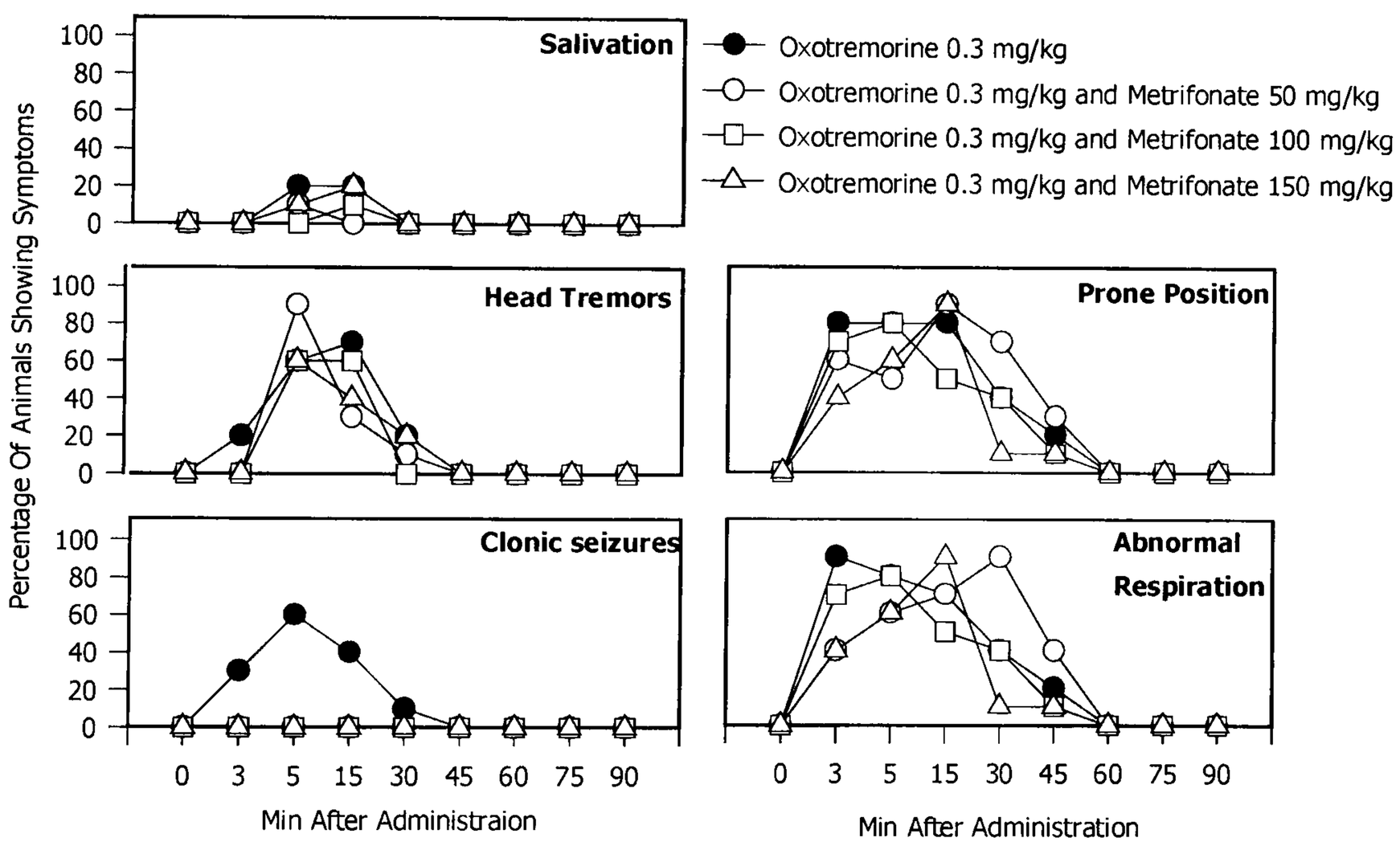


Figure 2: Effects of a challenge dose of oxotremorine 0.3 mg/kg in young adult rats subchronically treated with metrifonate 50, 100 or 150 mg/kg. The percentage of animals showing the symptoms was determined over an observation period of 90 minutes.

	Oxotremorine 0.3mg/kg	50 mg/kg	100 mg/kg	150 mg/kg
Salivation	4	1	1	3
Head Tremors	17	14	12	12
Prone position	30	30	25	21
Clonic seizures	14	0	0	0
Abnormal respiration	30	30	25	30

Table 1: Effects of a challenge dose of oxotremorine, 0.3 mg/kg (i.p) in young adult rats subchronically treated with metrifonate 50, 100 and 150 mg/kg. Entries represent the sums of observed symptoms during 9 observation points of 10 rats per group. All groups N = 10.

Sub-chronic metrifonate with nicotine

Adverse symptoms were observed in all animals 2 minutes after administration of nicotine alone or nicotine in combination with Metrifonate 50, 100 or 150 mg/kg (*see fig 3 and table 2*). No differences were observed between treatment groups averaged over all 9 observation points (*See table 2*). Symptoms included tachycardia ($F_{3,35}$= 0.01, n.s), ptosis ($F_{3,35}$= 0.94, n.s), Clonic seizures ($F_{3,39}$= 0.47, n.s), nausea ($F_{3,35}$= 0.09, n.s), prone position ($F_{3,35}$= 0.11, n.s) and nose bleeding ($F_{3,35}$= 0.35, n.s). The abnormal effects peaked about 2 and 15 minutes after administration of the challenge drug. They were transient and disappeared about 75 to 90 min after drug administration.

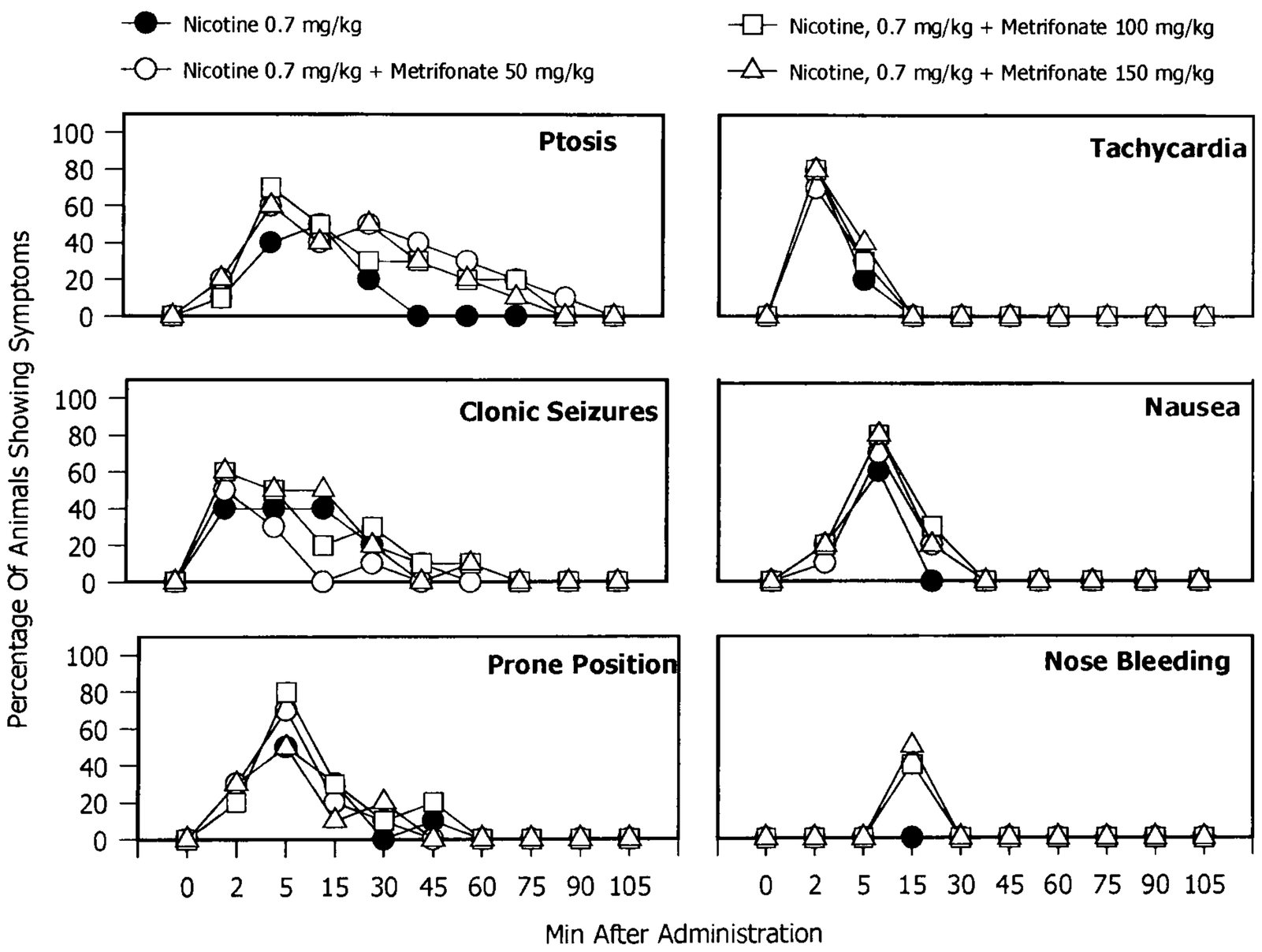


Figure 3: Effects of a challenge dose of nicotine 0.7 mg/kg in young adult rats subchronically treated with metrifonate 50, 100 or 150 mg/kg. Percentage of animals showing the symptoms over an observation period of 105 minutes is depicted.

	Nicotine 0.7 mg/kg	50 mg/kg	100 mg/kg	150 mg/kg
Tachycardia	9	10	11	12
Ptosis	12	19	23	23
Clonic Seizures	15	16	18	17
Nausea	8	10	13	12
Prone position	12	14	16	11
Nose Bleeding	0	4	5	4

Table 2: Effects of a challenge dose of 0.7 mg/kg nicotine (i.p) in young adult rats subchronically treated with metrifonate 50, 100 or 150 mg/kg. Entries represent the sums of observed symptoms during 9 observation points of 10 rats. All groups N = 10.

Sub-chronic metrifonate with Donepezil.

Adverse symptoms were observed in all animals 3 minutes after administration of donepezil or donepezil with Metrifonate 50, 100 or 150 mg/kg, particularly with the symptoms of Repetitive chewing and Hypoactivity (*See fig 4 and table 3*). Repetitive chewing ($F_{3,35}$ = 0.07, n.s) and Hypoactivity ($F_{3,35}$ = 1.64, n.s) were not differentially affected by the treatment over the 8 time points. However post hoc analysis revealed that the animals treated with 100 and 150 mg/kg metrifonate and donepezil showed more hypoactivity than the animals treated with donepezil alone. Head tremors were observed in the group treated with donepezil and Metrifonate 100 mg/kg ($F_{3,35}$ = 2.33, $p<0.01$). The abnormal effects peaked around 15 minutes after administration. They were transient and disappeared about 75 min after drug administration.

	Donepezil, 3 mg/kg	50 mg/kg	100 mg/kg	150 mg/kg
Repetitive chewing	13	14	13	16
Hypoactivity	19	18	28	21
Tremors	0	0	4	0

Table 3: Effects of a challenge does of donepezil 3 mg/kg in young adult rats subchronically treated with metrifonate 50, 100 or 150 mg/kg. Entries represent the sums of observed symptoms during 9 observation points of 10 rats. All groups N = 10.

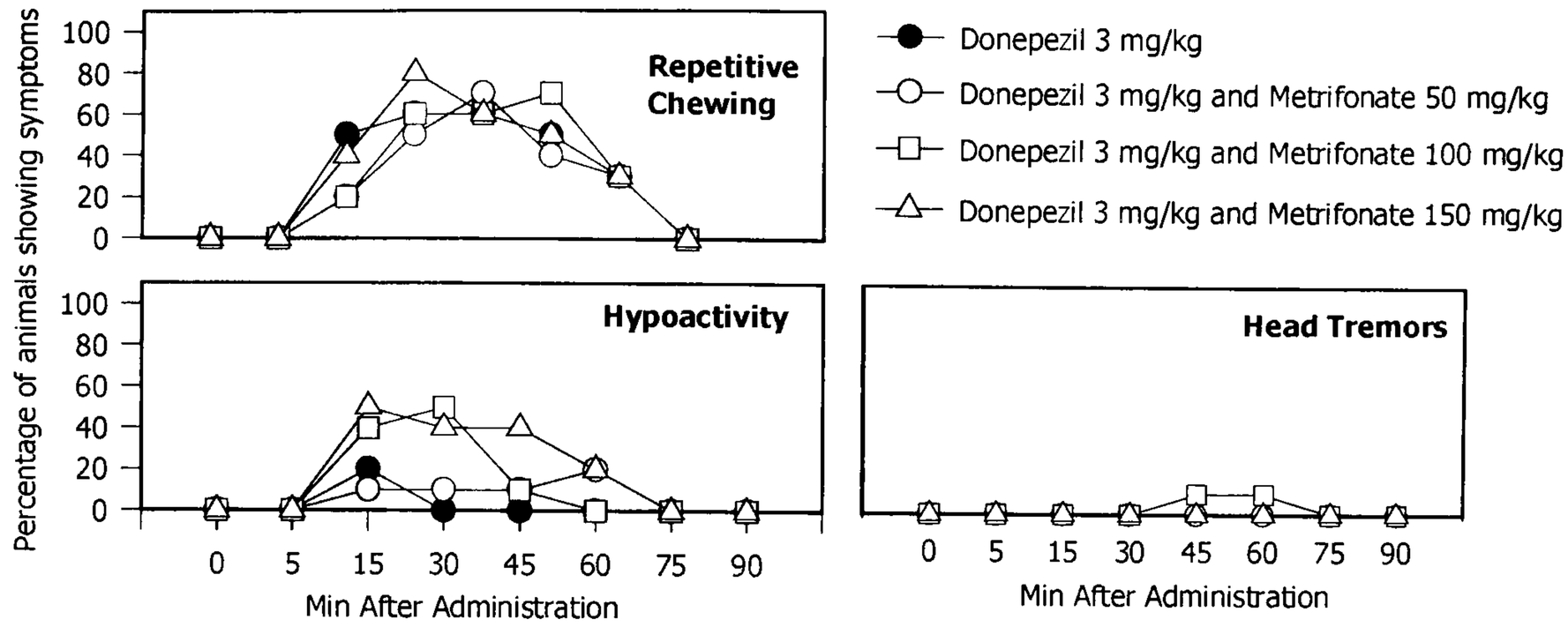


Figure 4: Effects of a challenge dose of 3 mg/kg donepezil in young adult rats subchronically treated with metrifonate 50, 100 or 150 mg/kg. Percentage of animals showing the symptoms over a period of 90 minutes.

Sub-chronic metrifonate with Bethanechol (See)

All animals exhibited adverse symptoms after administration of Bethanechol alone or in conjunction with Metrifonate 50, 100 or 150 mg/kg, particularly with the symptoms of Salivation and Exopthalmus (bleeding and swelling of the eye. (*See figure 5 and table 4*). Other symptoms observed were Kyphosis (hunched back) and Nose bleeding. Abnormal respiration was observed in 90-100% of all rats. This symptom occurred only at the 15 minute time point. No differences were observed between the bethanechol treated group and the groups treated with a combination of metrifonate and bethanechol. The abnormal effects peaked between 2 and 30 minutes after administration were transient by disappearing about 75 min after administration.

	Bethanechol 30 mg/kg	Metrifonate 50 mg/kg	Metrifonate 100 mg/kg	Metrifonate 150 mg/kg
Exopthalmus	26	27	27	23
Salivation	35	27	24	28
Kyphosis	20	17	18	18
Prone position	19	14	11	19
Nose Bleeding	5	3	2	3
Abnormal respiration	10	9	9	9

Table 4: Effects of a challenge dose of 30 mg/kg bethanechol, in young adult rats subchronically treated with metrifonate 50, 100 and 150 mg/kg. Entries represent the sums of observed symptoms during 8 observation points of 10 rats. All groups N = 10.

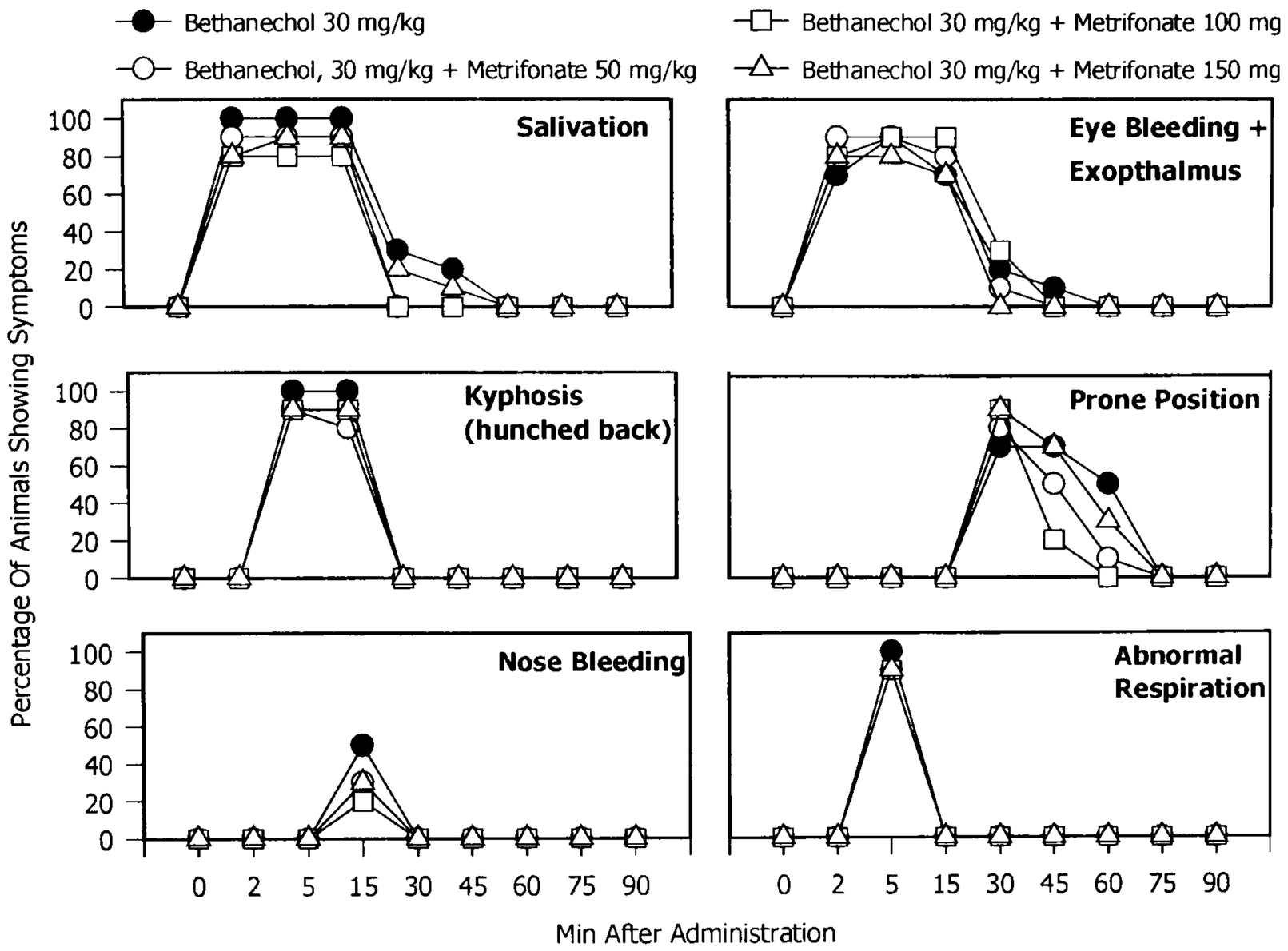


Figure 5: Effects of a challenge dose of bethanechol, 30 mg/kg in young adult rats subchronically treated with metrifonate 50, 100 or 150 mg/kg. Percentage of animals showing the symptoms over a period of 90 minutes.

Sub-chronic metrifonate with neostigmine

All animals exhibited adverse symptoms after administration of Neostigmine alone or in conjunction with Metrifonate 50, 100 or 150 mg/kg. 60-100% of rats showed symptoms, which included Ptosis, Prone position, Clonic seizures and slight tremor (*See figure 6 and table 5*). The 100 mg/kg metrifonate appeared to have a greater incidence of side effects over the 8 time point period compared to that of neostigmine, however further statistical analysis revealed no difference between treatment groups (Ptosis: $F_{3,35} = 0.05$, n.s; Prone Position: $F_{3,35} = 0.20$, n.s; Clonic seizures: $F_{3,35} = 0.09$, n.s; Tremor: $F_{3,35} = 0.33$, n.s). The abnormal effects peaked between 5 and 45 minutes after administration. They were transient and disappeared about 60 min after administration.

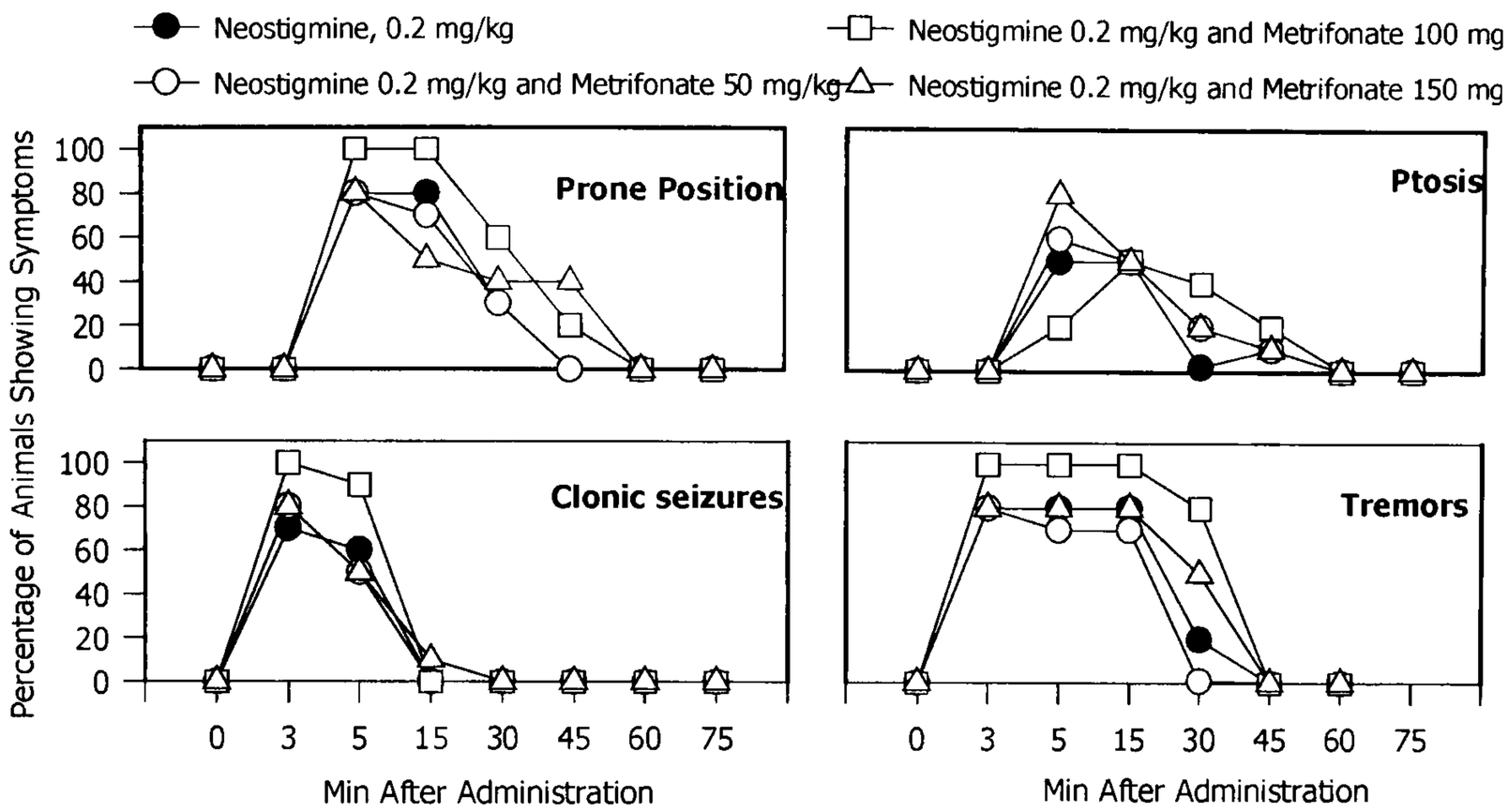


Figure 6: Effects of a challenge dose of 0.2 mg/kg neostigmine, in young adult rats subchronically treated with metrifonate 50, 100 or 150 mg/kg. The percentage of animals showing the symptoms over a period of 75 minutes is depicted.

	Neostigmine, 0.2 mg/kg	Metrifonate 50 mg/kg	Metrifonate 100 mg/kg	Metrifonate 150 mg/kg
Ptosis	13	14	13	16
Prone position	19	18	28	21
Clonic seizures	13	13	19	14
Tremors	26	23	38	29

Table 5: Effects of a challenge does of 0.2 mg/kg neostigmine, (i.p) in young adult rats subchronically treated with metrifonate 50, 100 and 150 mg/kg. Entries represent the sums of observed symptoms during 8 observation points of 10 rats. All groups N = 10.

2.3: Hypothermic response to cholinergic compounds in rats with sub-chronic metrifonate

This experiment investigated whether challenge doses of 0.7mg/kg nicotine, 0.3 mg/kg oxotremorine or 3 mg/kg donepezil affected the body temperature of rats treated daily for 12 weeks with metrifonate 50, 100 or 150 mg/kg.

Procedure

Animals: The subjects were 38 male HsdCpb:WU (Harlan Wistar) rats (supplied by Harlan-Winkelmann, Borchen) with free-feeding body weights ranging 220- 250 grams aged 10 weeks at the start of the experiment. Rats were housed in pairs in standard Makrolon™ Type III cages in which water was continuously available, with a 12:12 hour light/ dark cycle, lights on at 07.00 am.

Apparatus and Methods: Immediately preceding the administration of the challenge drugs the body temperature was measured electronically (Testotherm 9019, Testotherm GmbH, Lenzkirch, Germany) with a rectal probe. Fifteen, 30, 45, 60, 90, 120, 180 and 240 min after administration of the challenge doses, additional measurements were performed.

Analysis: The effects of the compounds were analysed with a Treatment by Time ANOVA, in which the time after administration was considered a repeated measures factor.

Cholinesterase Inhibition:

Methods and Analysis The inhibition of cholinesterase activity was measured and analysed as with the brain samples in section 9.1

Results

Donepezil (See figure 7, panel A)

Averaged over the entire observation period (3 hrs) drug treatment affected body temperature differently (GENERAL MEAN: $F_{3,34}= 7.51$, $p<0.001$). Post hoc analyses on the general mean by Fischer's LSD revealed that the groups treated with combination of donepezil and 100 or 150 mg/kg metrifonate produced a higher increase in body temperature than the control group treated with donepezil alone. The body temperature changed over the time of observation (TIME: $F_{8,272}= 60.15$, $p<0.001$) and this change in body temperature was different between treatment groups (TIME by TREATMENT: $F_{24,272}= 4.35$, $p<0.001$). All groups returned to pre-treatment body temperatures after 3 hours (ANOVA on last time point:$F_{3,34}= 0.77$, n.s).

Administration of donepezil to the group treated with 100 mg/kg metrifonate produced the highest increase of body temperature 45 min post administration (37.98 °C ± SEM).

Nicotine (see figure 7, Panel B)

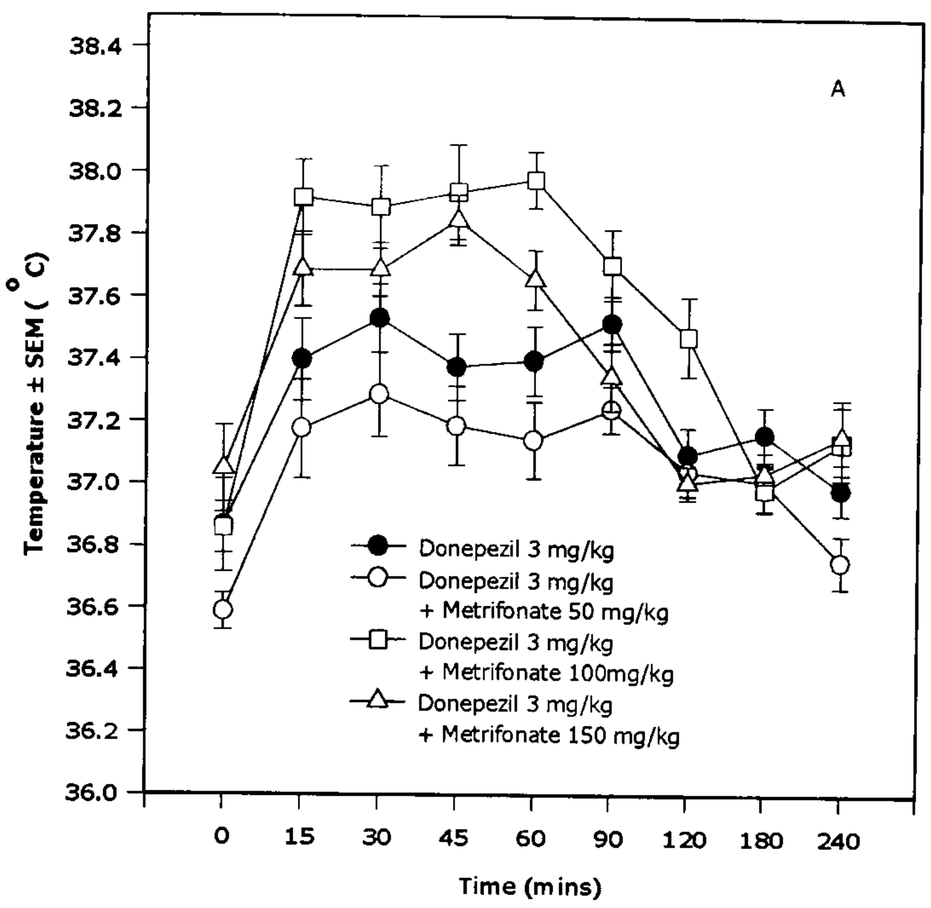
One animal was removed from analysis

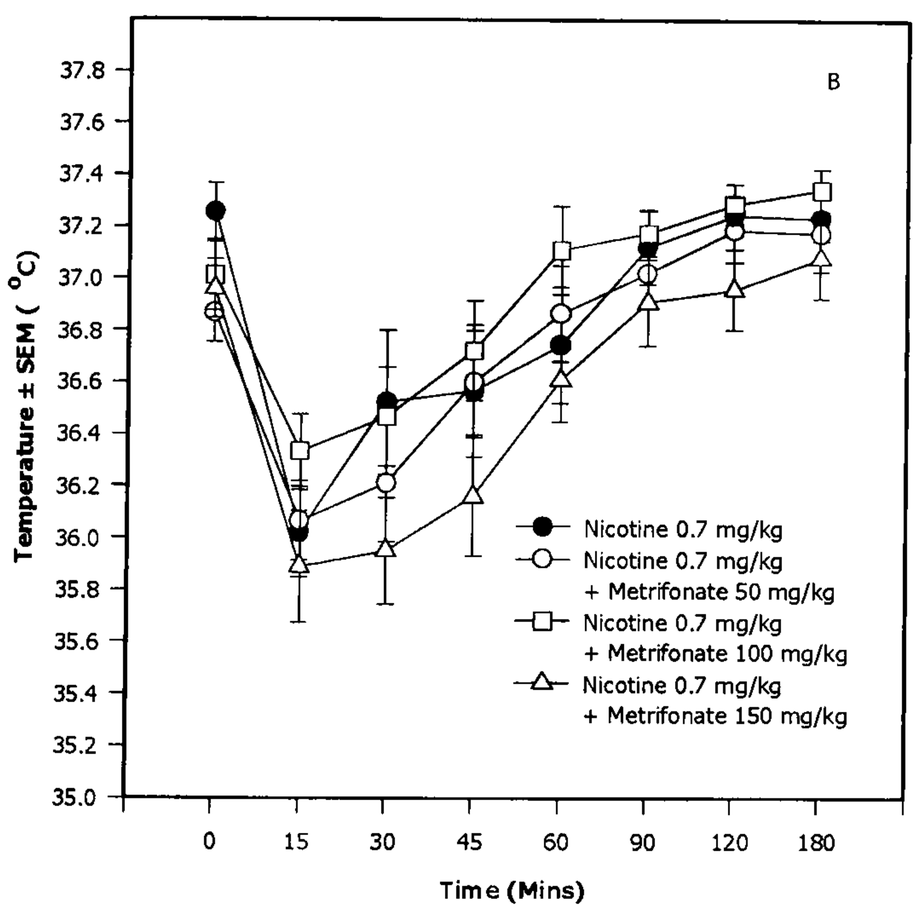
Nicotine appeared to affect the body temperature of control (nicotine 0.7 mg/kg + vehicle) and metrifonate treated (nicotine + 50, 100 or 150 mg/kg metrifonate) rats differently when averaged over the entire observation period (180 mins). However, this impression was not confirmed statistically (GENERAL MEAN:$F_{3,33}$= 1.52, n.s). The body temperature changed over the time of observation (TIME: $F_{8,272}$= 57.39, $p<0.001$), however this change in body temperature was not different between treatment groups (TIME by TREATMENT: $F_{24,272}$= 0.96, n.s). All groups returned to pre-treatment body temperatures after 3 hours (ANOVA on last time point: $F_{3,33}$= 1.54, n.s).

Oxotremorine (See figure 7, Panel C)

Averaged over the duration of the observation period (180 mins) oxotremorine did not differentially affect the body temperature of control (oxotremorine 0.3 mg/kg + vehicle) and metrifonate treated (oxotremorine + 50, 100 or 150 mg/kg metrifonate) rats (GENERAL MEAN: $F_{3,34}$= 0.09, n.s). The body temperature changed over the time

of observation (TIME: $F_{8,272}$= 20.39, $p<0.001$), however this change in body temperature was different between treatment groups (TIME by TREATMENT: $F_{24,272}$= 2.66, $p<0.001$). All groups returned to pre-treatment body temperatures after 3 hours (ANOVA on last time point: $F_{3,34}$= 0.21, n.s).





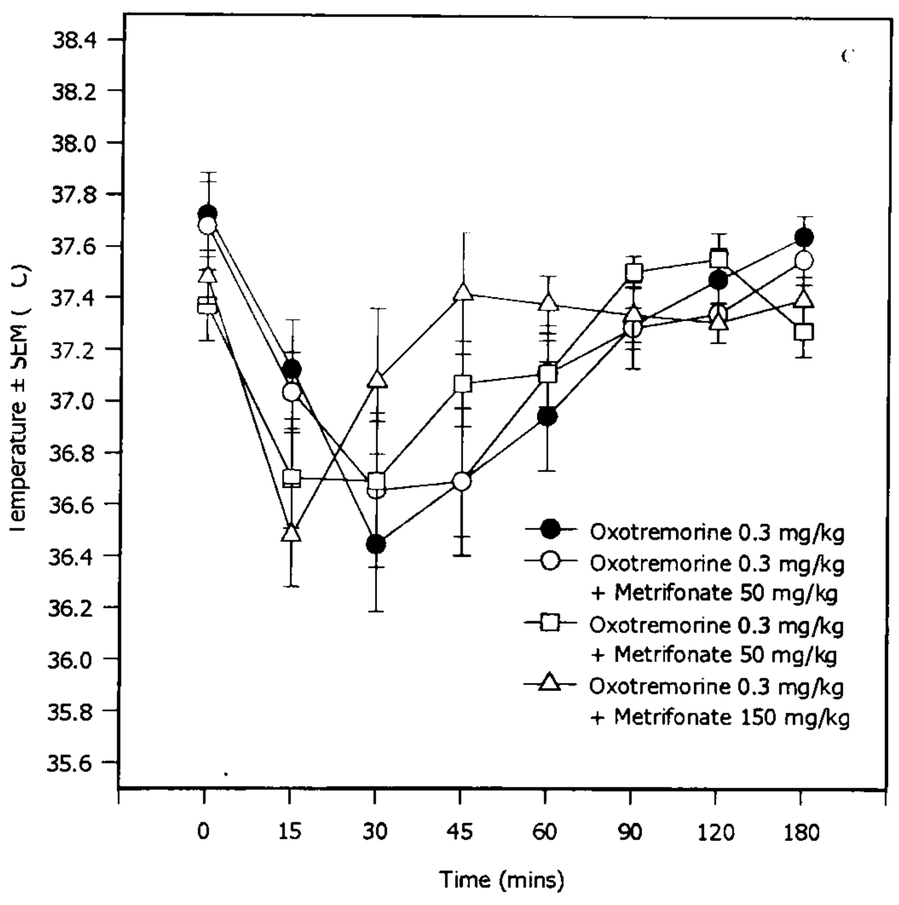


Figure 7 Panel A: Effects of 3 mg/kg Donepezil on body temperature in Wistar rats pre-treated with metrifonate 50, 100 and 150 mg/kg daily for 12 weeks. *Panel B*: Effects of 0.7 mg/kg Nicotine on body temperature in Wistar rats pre-treated with metrifonate 50, 100 and 150 mg/kg daily for 12 weeks. *Panel C*: Effects of 0.3 mg/kg Oxotremorine on body temperature in Wistar rats pre-treated with metrifonate 50, 100 and 150 mg/kg daily for 12 weeks. Results are means ± SEM

Cholinesterase Inhibition

Averaged over all groups the brain cholinesterase activity after 12 weeks was reduced by metrifonate treatment ($F_{3,35}$ = 5.95, $p<0.05$). Post hoc comparisons revealed that both the groups treated with 100 and 150 mg/kg metrifonate differed from vehicle treated group, producing a 33 and 50 % reduction in Cholinesterase activity, respectively.

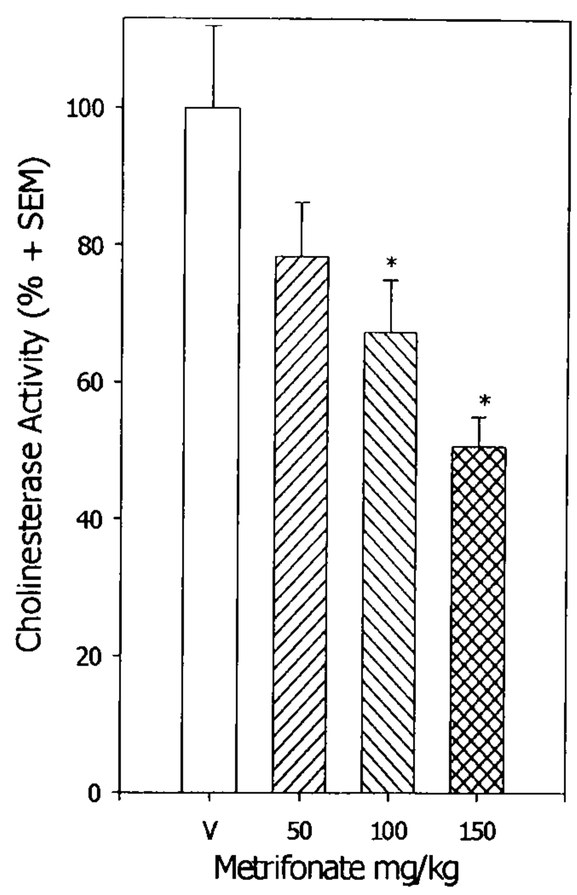


Figure 8: Effects of sub-chronic Metrifonate on cholinesterase activity in the brain. Data is measured as a percentage of control animals, ± SEM.

Discussion

The aim of the present study was to assess whether long-term inhibition of cholinesterase activity by metrifonate created adaptive changes in the development of tolerance. In addition, the question was addressed whether the adaptive changes were a central or peripheral phenomenon. To do this the cholinesterase activity after sub chronic metrifonate treatment was measured in tissues which produce the cholinergic side effects of salivation, tremor diahorrea etc. Changes to the adverse event profile and frequency with peripherally and centrally acting nicotinic, muscarinic and cholinesterase stimulation and the effects on body temperature after central stimulation were assessed.

Cholinesterase Activity

In experiment 9.1, Cholinesterase inhibition occurred in a dose dependent manner. Metrifonate treatment caused a 30 % reduction in brain cholinesterase activity by the highest dose tested (50 mg/kg). Erythrocyte cholinesterase inhibition after 12 weeks of treatment as compared to the shorter treatment period of 6 weeks mediated the same level of inhibition (i.e. 60% inhibition). The concomitant inhibition of erythrocyte and brain cholinesterase activity up to three months of treatment confirms the findings of Moriearty *et al* (1991) that metrifonate does not induce any alterations in normal blood cell differentiation. Peripheral inhibition of cholinesterase activity such as on erythrocytes is thought by some to be unwanted and may constitute a safety risk, and that brain selective cholinesterase inhibitors would be more preferable. However, as shown in this study, pharmacologically active doses of metrifonate after long term administration, inhibition of cholinesterase activity in brain and blood was well tolerated by rats. Further findings of this study are that no significant inhibition in other peripheral tissues such as salivary glands, skeletal and smooth muscle were observed. It appears that inhibition of peripheral cholinesterase in the blood does not necessarily lead to peripheral side effects.

Adverse event profile and body temperature

Repeated administration of cholinesterase inhibitors is thought to induce behavioural tolerance to the transient, but supposedly limiting cholinergic side effects. Compared to an acute administration of metrifonate to drug naive animals, the same challenge dose of metrifonate given to subchronically pre-treated rats results in a lower incidence of adverse effect (Blokland *et al,* 1995). The 50mg/kg dose was chosen because it is effective in patients (Cummings et al, 1998) and behavioural tasks in animals (Schmidt et al, 1997) The 150mg/kg dose of metrifonate was chosen as an outer limit to the development of tolerance over time, higher concentrations would cause irreversible damage or even death.

Behavioural tolerance is supposedly due to the activation of secondary processes which compensate for the increase in acetylcholine resulting from cholinesterase inhibition In view of this, challenge doses of either centrally or peripherally acting

muscarinic, nicotinic and cholinesterase inhibitors were administered to rats subchronically treated with metrifonate.

No distinct changes were observed with the peripherally acting compounds bethanechol (muscarinc agonist) or neostigmine (cholinesterase inhibitor). In response to oxotremorine treatment, a centrally acting muscarinic agonist, there was a subsensitivity to muscarinic receptor-mediated changes in only one of the observed side effects i.e. clonic seizures whereas nicotine, caused no changes in sensitivity an rats pre-treated with metrifonate. These two compounds also produced no changes in sensitivity in body temperature, which is regulated by muscarinic receptors in the hypothalamus (Ghanta et al, 1996). Donepezil, a centrally acting cholinesterase inhibitor produced a supersensitivity, in both adverse effects and body temperature. Lack of effects with muscarinic and nicotinic agents and a supersensitivity to cholinesterase inhibitors suggests possible changes to the cholinesterase enzyme itself rather than changes in acetylcholine receptors.

Previous studies have shown tolerance development with the repeated administration of cholinesterase inhibitors (Chippendale *et al*, 1972; Overstreet *et al*, 1974; Costa *et al*, 1982; Russel *et al*, 1986). This effect was interpreted as resulting from a down regulation of muscarinic receptors in the brain and other tissues (Churchill, *et al* 1984; Costa *et al*, 1982; Bushnell *et al*, 1991). Pharmacological evidence for tolerance to chloropyrifos was observed as a reduction in sensitivity to the hypothermic response of oxotremorine providing a functional index to muscarinic receptor down regulation (Bushnell *et al*, 1994). Development of tolerance has also been observed during repeated exposure to di-isopropylfluorophosphate (DFP) (Overstreet *et al*, 1974; Bushnell *et al*, 1991) and disulfoton (Costa *et al*, 1982). However, in the case of metrifonate no changes in either muscarinic or nicotinic receptor binding or affinity are observed after subchronic treatment (Hinz *et al*, 1998). This study has shown no changes to the hyperthermic response and side effects. Consequently another mechanism must be responsible for the changes in tolerance to metrifonate treatment.

One such mechanism could be changes in the second messenger systems within the cell. Past studies have looked at the effects of cholinesterase inhibitors on

muscarinic second messenger systems. An important receptor linked signal transduction system for the muscarinic receptors involves the hydrolysis of membrane phosphoinositides (PI) to diacylglycerol and inositol triphosphate, both of which may act as second messengers (for review see Ehlert *et al*, 1980)

Experiments concerning repeated cholinesterase inhibition on muscarinic receptor coupled PI hydrolysis have been inconsistent (Costa *et al*, 1986) found that disufoton (an OP compound) decreased carbachol-stimulated PI hydrolysis in rat brain cortical slices. This was also observed after administration of DFP (Pintor *et al* 1992). However, Abdallah and El-Fakahany (1991) reported no changes in hydrolysis in the rat cortex, hippocampus or striatum after repeated DFP administration. Further, Keifer-Day and El-Fakahany (1992) reported no change in muscarinic receptor agonist stimulated PI hydrolysis in brain cells from mice treated with tacrine (a cholinesterase inhibitor). It would be important to determine whether metrifonate is affecting this second messenger system rather than producing changes in receptor density and affinity.

The cholinesterase (ChE) enzyme can be subdivided into heteromeric and homomeric families as well as into globular and asymmetric forms (See Chapter 1, pg 3-4). This diversity is produced by alternative mRNA splicing. DFP has been shown to have more affinity for the intracellular membrane bound G_1 form of AChE and metrifonate has been shown to have similar affinity for both the G_1 and extracellular membrane bound G_4 form (Ogane et al, 1992). These differences might be related to an effect on the allosteric binding site or on a non-catalytic binding site, which is known to have a regulatory effect on enzyme activity (Gentinetta, et al, 1976; Skau, 1986). This may explain that metrifonate and DFP may have differences in their eventual effects on muscarinic receptor binding and affinity.

Cholinesterase can be found as one of two forms, acetylcholinesterase (AChE) or butyrylcholinesterase (BuChE). A possible explanation for the effects of the increased sensitivity to donepezil could be the affinity for the compounds for either AChE or BuChE. According to published data donepezil is more selective for AChE (Rogers et al, 1991) and metrifonate has more affinity for BuChE than for AChE.

(Pacheco *et al*, 1995). The potency and respective selectivity index for AChE versus BuChE are not related to their tolerability and as such more selective BuChE compounds such as metrifonate do not cause more symptoms than inhibitors that are selective for AChE, i.e. donepezil (Schmidt & van der Staay, 1998) when administered alone. However, the combination of the two compounds may cause an additive effect, thus increasing the sensitivity to side effects and body temperature. This needs to be studied further with other combinations of AChE and BuChE selective compounds to see if this is a general effect or if this effect is specific for metrifonate alone. Further studies on the distribution of the compounds within different regions of the brain, with also studies in abundance and distribution of AChE and BuChE may further explain this effect.

Finally, the administration of metrifonate may counter regulate changes in the expression of the genes coding for AChE and BuChE in the brain (See chapter 10).

In conclusion from this study it is thought that the development of tolerance is a centrally acting phenomenon that is mediated by either changes of the cholinesterase enzyme within the brain or of the gene expression of AChE and BuChE.

Changes in Gene Expression of Acetyl and Butyrylcholinesterase in Brain after Sub-chronic Metrifonate Treatment

Abstract

A modified polymerase chain reaction (PCR) assay was used to analyse acetyl and butyrylcholinesterase gene expression in the rat brain after sub-chronic treatment with metrifonate (10, 30, 50 and 100 mg/kg). The method describes the use of the housekeeping enzyme hypoxanthine phosphoribosyltransferase (HPRT) as an internal standard, since its range of detectable expression is similar to that of the enzymes under test.

Results show that sub chronic treatment with metrifonate produced about a 2-fold increase in both AChE and BuChE mRNA expression.

This up regulation of ChE expression could be responsible for the decrease in adverse side effects observed over time (see chapter 9) and would generally indicate a decrease in the therapeutic efficacy of AChE inhibitors in Alzheimer therapy. However, there are no changes in the cognitive benefit of metrifonate in patients (Dubois et al., 1999) or in experimental animals (Schmidt et al., 1997). At this time it is unknown as to the reason of these effects though it is thought that possibly another mechanism within the brain maintains the cognitive improvement.

Introduction

Metrifonate is a dual inhibitor of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) developed for the cholinergic therapy of Alzheimer's disease (Schmidt and Heinig, 1998; Jann, 1998). Its long-term efficacy has been demonstrated in double-blind, placebo-controlled clinical trials (Becker et al., 1996; Morris et al., 1998; Dubois et al., 1999). The compound also improves the cognitive behavior of animals (Schmidt et al., 1997) whereby the best results are usually obtained in the dose range of 10 to 50 mg/kg orally.

Cholinesterase inhibitor treatment produces an accumulation of acetylcholine at the muscarinic and nicotinic cholinergic receptors; this produces classical cholinergic side effects such as salivation, diarrhoea, tremor etc. Multiple doses of some cholinesterase inhibitors induce the development of tolerance, which is shown by a marked recovery from the signs of toxicity associated with cholinergic overstimulation (Chippendale *et al*, 1972; Overstreet *et al*, 1974; Bigami *et al*, 1975; Costa, *et al*, 1982; Russel *et al*, 1986).

The resulting subsensitivity is not restricted to the occurrence of cholinergic side effects, but may also affect the functionality of central cholinergic pathways including those involved in attentional or cognitive processes (e.g. Bushnall *et al*, 1991; McDonald *et al*., 1988; Stamper *et al*., 1988; Wolthuis *et al*, 1990). However, in the case of metrifonate one behavioural aspect of cholinergic activation, i.e. adverse reaction to cholinergic overstimulation becomes hyposensitive, the other, cognitive improvement is spared.

As AChE is a vital enzyme, a long-term inhibition might result in a counterregulatory upregulation of the expression of the gene coding for this enzyme to restore normal levels of activity. This would, however, decrease the therapeutic efficacy of AChE inhibitors in Alzheimer therapy and necessitate an increase in dosage over time. In order to assess whether there are changes in gene expression, we treated rats for three months with metrifonate (0, 10, 30, 50 or 100mg/kg orally twice daily) and measured the levels of AChE and BuChE expression in whole forebrain samples.

Procedure

Animals: Male HsdCpb:Wu Wistar rats, weighing about 220 to 250 grams, were obtained from Harlan-Winkelmann, Borchen, Germany. They were allowed to adapt to our animal facilities for one week before start of the pretreatment. The rats were housed in groups of two in standard Makrolon™ Type III cages. Food and water was continuously available, with a 12:12 hour light/ dark cycle, lights on at 07.00 am.

Drug Administration: Rats (N= 7-8) were pre-treated orally twice daily with 10, 30, 50 or 100 mg/kg Metrifonate, in an application volume of 5 ml/kg for 12 weeks in order to induce steady state cholinesterase inhibition. Controls received the vehicle (Sodium citrate buffer, pH 5.5) under identical conditions.

Analysis: Gene expression of AchE and BuChE across the selected dose range was analysed by Kruskal-Wallis, with the factor Dose, complemented by post-hoc Fischer LSD comparisons.

Determination of AChE and BuChE gene expression in brain

RNA preparation (the method of Chromczynski and Sacchi (1987) was used)

18 hours after the final application the animals treated with metrifonate or vehicle were killed by decapitation. Whole brains were dissected over ice and homogenised for 30-60s in 5 ml of lyses buffer. Aliquots of 1ml were then prepared. To this, 50 µl 2 M NaAc pH 4,0; 500 µl Phenol and 100 µl Chloroform/Isoamylalcohol 24:1 was added, and then centrifuged for 20 min at 4°C and 14000 rpm. The top layer was removed. 8-15 µl RNA-Matrix was then added and vortexed for 30s, followed by 5 min agitated incubation at 55°C (Thermomixer, Eppendorf). Following 30s centrifugation at 14000 rpm, 250 µl RNA-Wash solution was added and then re-centrifuged for an additional 30s

The top layer was removed and the pellet allowed to dry, preferably overnight. 20-30µl DEPC- H_2O were added and agitated for 5 min at 55 °C. Following centrifugation for 30 s at 14000 rpm the top layer was removed and re-centrifuged twice to make sure the entire matrix was removed. The RNA was stored at –70°C.

Reverse transcription

RNA was reverse transcribed into cDNA as described by Murphy et al. (1993). A 40µl reaction mixture contained about 200 ng - 2 µg of total RNA.

A Mastermix was first prepared without RNAsin (RNAase inhibitor, Gibco) and RT (Reverse Transcriptase, Gibco), over ice (See *table 1*). Once these were added the mix was incubated for 10 min (annealing) and then for 1 h at 37°C (synthese). Denaturing the enzymes then followed for 3 min at 96°C. The samples were then held at –20°C until required.

Compounds	Volume
5 x buffer	8 µl
dNTP (25 mM)	4 µl
Oligo dTPrimer (500 µg/ml)	1 µl
DTT (0,1 M)	1 µl
DEPC-H_2O,	3-15 µl
RNAsin (10000 U/ml)	1 µl
RT (Superscript, 200 U/µl)	2 µl
RNA	8-20 µl

Table 1: Mastermix for the reverse transcription of RNA to cDNA.

Primers and control fragment (see table 2)

Primer pairs for the 5' and 3' region were selected. The annealing temperature was optimised empirically for each primer pair. Primer pairs were designed such that they do not exhibit 3'-complementarity and span one or more intones to distinguish amp icons of cDNA from those of genomic DNA. As control fragment for hypoxanthine phosphoribosyltransferase (HPRT) a synthetic gene containing 5' and 3′ primers were constructed using complementary 40mer oligonucleotides chosen with the GENMON-program (design and use of primers and probes is reviewed in O'Garra and Vieira, 1992) by TIB Molbiol, GmbH iG, Berlin. The gene was cloned in pBluescript II KS+ (Stratagene, La Jolla, CA) and controlled for the correct sequence. It was obtained as fragment from the plasmid by restriction with Pst I/Kpn I. The specificity of the PCR products was verified by restriction analysis, using two restriction enzymes indicative of the expected amplified sequence.

Table 2: Rat gene primer sequences

Gene	Primers	Gene Sequence
HPRT	sense:	5'-GTA ATG ATC AGT CAA CGG GGG AC-3'
	antisense:	5'-CCA GCA AGC TTG CAA TAA CCA-3'
	probe	5'-GCT TTC CCT GGT TAA GCA AGT CAG CCC C-3'
AChE	sense:	5′-CAG GCC TAC TTC TCC CAC AC -3′
	antisense:	5′-GAG TCA CTG ACA TCG GGT CT -3′
BuChE	sense:	5′-AGT GGA TGG CGA TTT TCT CAC-3′
	antisense:	5′-CCA GTG CAG GGC AGA TGA T-3′

Competitive RT-PCR

The competitive PCR has been introduced (Gilliland 1990) in which a competitor control fragment is amplified together with sample cDNA in the PCR reaction

mixture with the same primers. Variations in efficiency of amplification between reactions can be estimated using the control fragment as internal control. The control and sample PCR products are distinguished by differences in length. With the known input concentration of the control fragment and amplification of both PCR products occurring proportionally, the sample cDNA can be quantified. This method was used to quantify HPRT (housekeeping gene content of cDNAs).

HPRT cDNA quantification was performed as described by Siegling et al. (1994). A cDNA equivalent of about 5 ng total RNA was amplified in a 25 µl reaction volume containing 250 µM of each dNTP, 100 µM of the appropriate primer pair, 2.5 µl 10-fold PCR buffer (Perkin Elmer/Cetus, Emeryville, CA) and 0.5 µl Taq DNA polymerase ("Ampli"-Taq, Perkin Elmer/Cetus). After an initial denaturation step, cDNA samples were subjected to rounds of denaturation (94° C for 15 sec), annealing (68° C [beta-actin] and 60° C [AChE, BuChE] for 15 sec) and extension (72°C for 15 sec) using the thermal cycler 9600 (Perkin-Elmer/Cetus). According to the varying contents of specific cDNA and varying amplification efficiencies, the samples were subjected to 30 cycles: Control PCRs without cDNA were performed in all experiments to exclude contamination. To correct for variations across different preparations, the cDNA samples were adjusted to equal input concentrations based on their HPRT content before determining cytokine cDNA concentrations. PCR products were subjected to agarose (1%) gel electrophoresis, stained with SybrGreen® (Biozym) and the intensity of the beta-actin bands was measured using a video imaging system (Herolab) with the appropriate software.

TaqMan-Polymerase chain reaction (PCR)

cDNA quantification was performed as described in the user manual. A HPRT-cDNA amount of about 10-20 pg was amplified in a 25 µl reaction volume of the TaqMan PCR CORE Reagent Kit N808-0228 (Perkin Elmer/Cetus, Emeryville, CA) and a cDNA amount of about and 30-300 fg (AChE), 3-30 fg (BuChE) was amplified in a 25 µl reaction volume of the SYBR Green PCR Core Reagent Kit 4304886 (Perkin Elmer/ Cetus, Emeryville, CA) using the sequence detection system 1.6.3 (PE Applied Biosystems). To correct for variations across different preparations, the HPRT content of the specific cDNA samples was determined.

According to the varying input concentrations of cDNAs AChE/BuChE amounts were quantified relative to their HPRT content. Control PCRs without cDNA were performed in all experiments to exclude contamination. To avoid unspecific products AChE/BuChE-PCR products were subjected to agarose (1%) gel electrophoresis, stained with SybrGreen® (Biozym).

Results

Treatment effects on AChE and BuChE gene expression in brain

Adjustment of HPRT content of cDNAs:

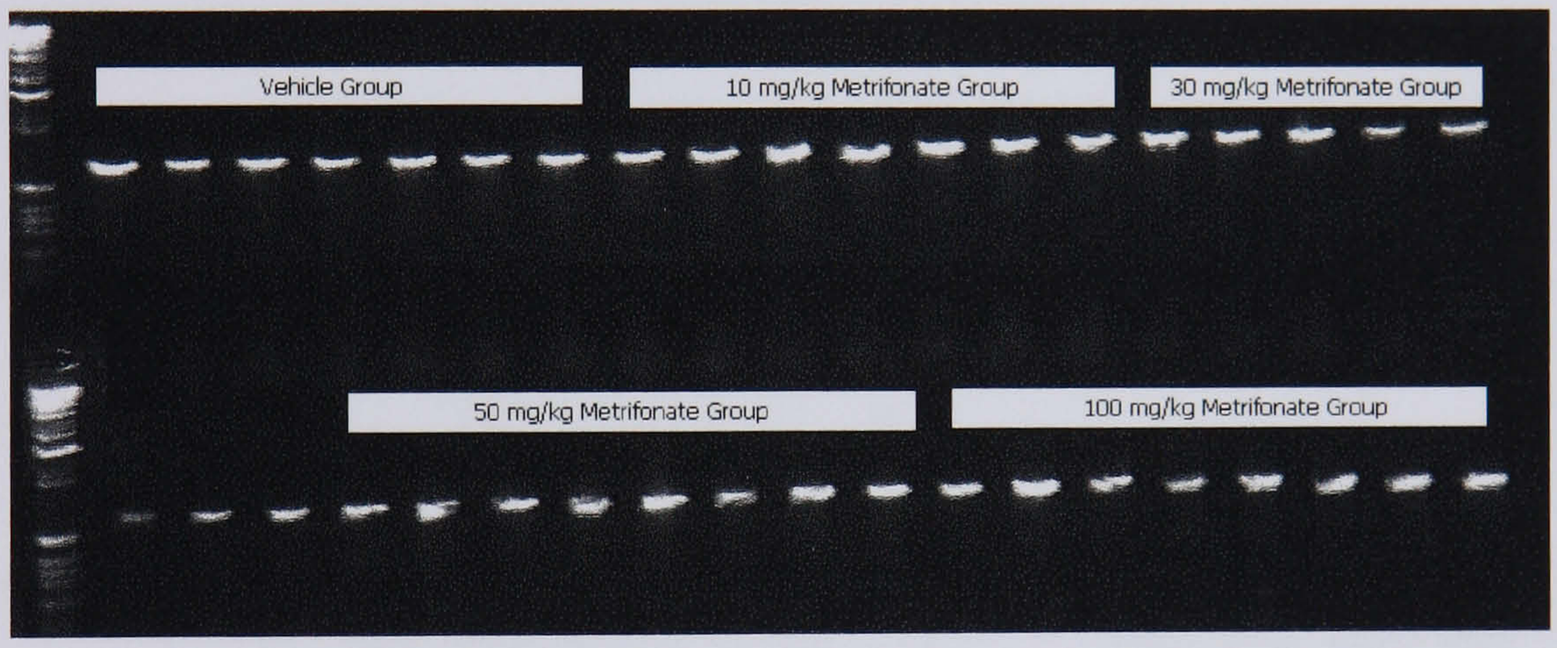


Figure 1: Initial analysis of HPRT expression in forebrain samples of rats treated sub-chronically with metrifonate (10, 30, 50 and 100 mg/kg). Arbitrary units for HPRT levels in the samples were obtained from a standard curve (*not shown*).

AChE and BuChE Standards:

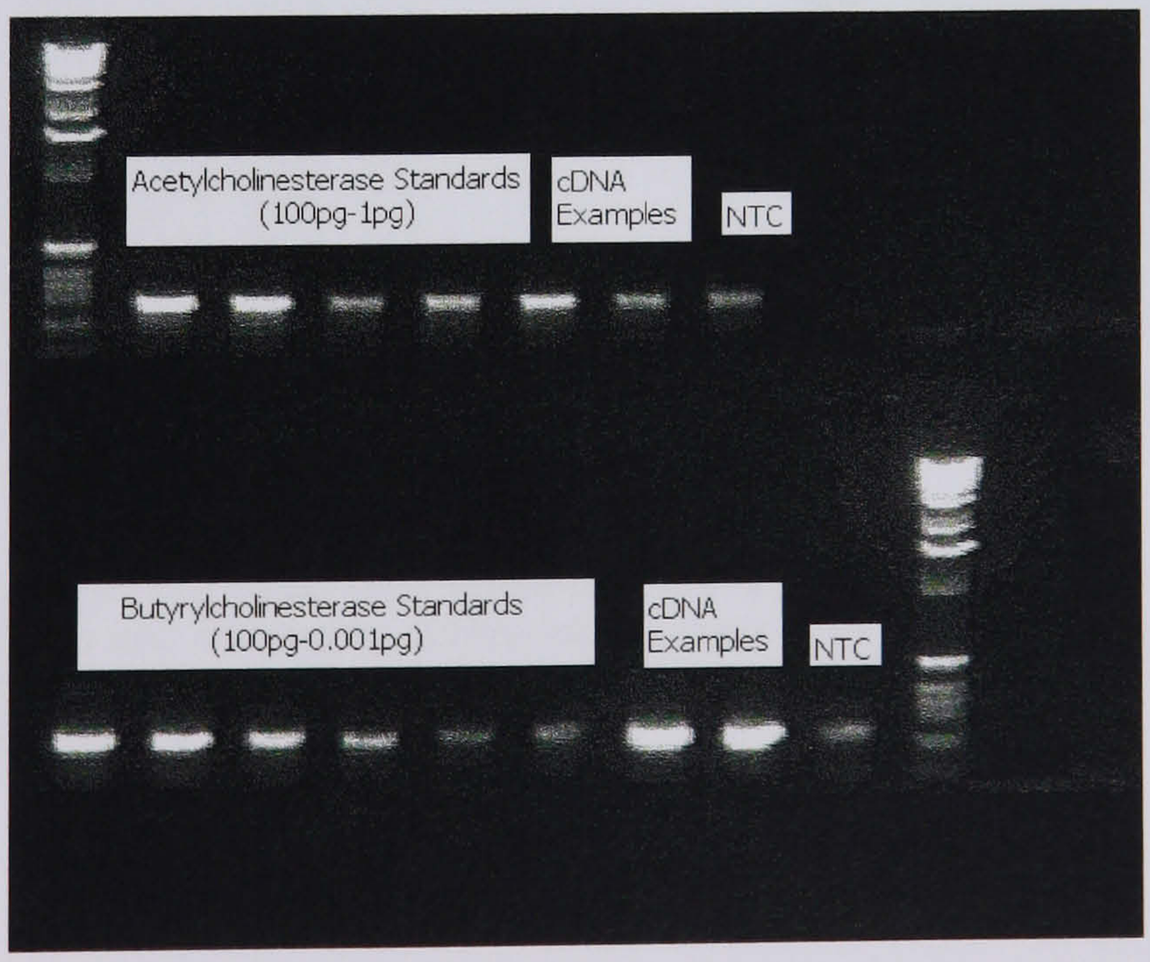


Figure 2: Initial analysis of *AChE and BuChE* in forebrain samples of rats treated sub-chronically with metrifonate (10, 30, 50 and 100 mg/kg) for quantification of AChE and BuChE gene expression.

Quantification of AChE- and BuChE-gene expression using TaqMan-PCR technique:

Sub-chronic treatment of metrifonate produced an increase in AChE mRNA expression (Kruskal-Wallis: 8.847, $p<0.05$). Further post hoc analysis revealed that the dose of 50 mg/kg metrifonate produced a 2-fold increase compared to vehicle treated animals.

BuChE mRNA expression was also shown to increased after sub- chronic metrifonate treatment (Kruskal-Wallis: 11.48, $p<0.05$). Post hoc analysis revealed that both the 30 and 50 mg/kg doses differed from vehicle.

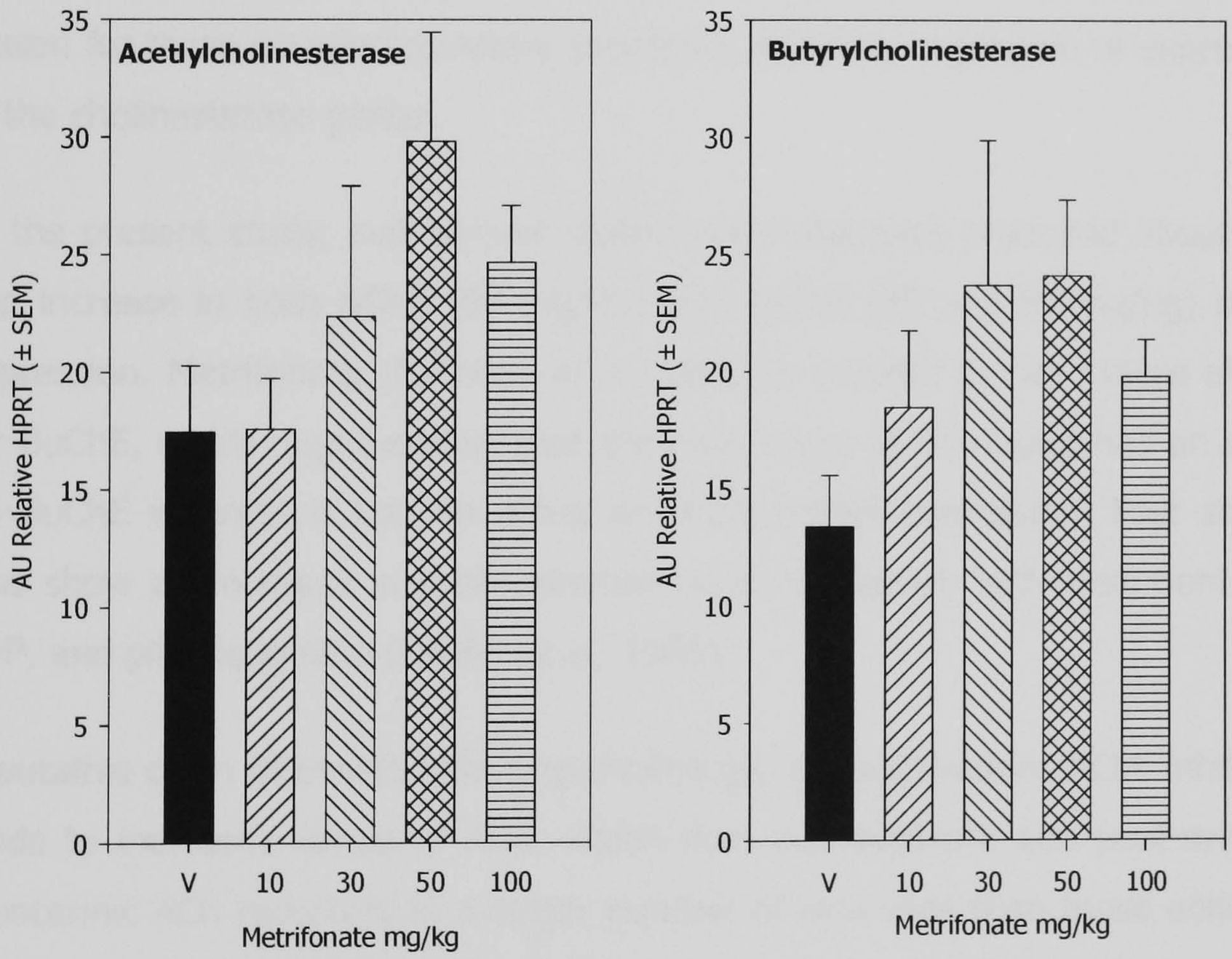


Figure 3: mRNA expression of AChE (*Left Panel*) and BuChE (*Right Panel*) in brain following chronic Metrifonate (10, 30, 50 and 100 mg/kg administration, twice daily p.o., for 12 weeks to Wistar rats compared to controls (Sodium Citrate buffer, pH 5.5). Data is presented as the mean of arbitrary units relative to HPRT concentration + SEM.

Discussion

Summary of results

Inhibitors of cholinesterases are being explored for the treatment of Alzheimer's disease. The aim is to rapidly enhance the levels of synaptic acetylcholine through inhibition of the hydrolytic capacities of cholinesterase's. Multiple doses of some cholinesterase inhibitors induce the development of tolerance, which is shown by a marked recovery from the signs of toxicity associated with cholinergic overstimulation (Chippendale *et al*, 1972; Overstreet *et al*, 1974; Bigami *et al*, 1975; Costa, *et al*, 1982; Russel *et al*, 1986) supposedly due to the activation of secondary processes which compensate for the increase in ACh resulting from cholinesterase inhibition. One possible reason for these counterregulatory processes is the up regulation of expression of the cholinesterase genes.

In the present study, sub chronic metrifonate treatment produced about a 2-fold increase in both AChE (50 mg/kg) and BuChE (30 and 50 mg/kg) mRNA expression. Metrifonate (Pacheco *et al*, 1995) is thought to have more affinity for BuChE, which might explain that the lower dose of 30 mg/kg had an effect on BuChE whereas it had no effect on AChE mRNA expression. Past studies also show an increase in cholinesterase gene expression with treatment with DFP, and physostigmine (Kaufer et al, 1999),

A putative chain of events following cholinergic hyperactivation: AChE inhibition leads to increased levels of ACh, which then activates pre and post-synaptic muscarinic ACh receptors in a larger number of neurones than those activated under normal conditions. This increase in number of neurons firing action potentials leads to increased population spike amplitudes in response to constant electrical stimulation. The consequent depolarisation and Ca^{2+} influxes eventually cause, via Ca^{2+} responsive elements (CRE) in the c-fos promoter, elevated c-fos mRNA levels (Ghosh et al, 1994). This in turn modulates the transcription of down stream genes encoding for AChE, ChAT and the vasicular ACh transporter (Kaufer et al, 1999). The results then suggest a feedback mechanism by which the AChE gene is activated by cholinergic transmission leading to increased AChE protein and

accelerated degredation of acetylcholine at cholinergic synapses (Nitsch et al, 1998).

Significance for AD therapy

Counterregulation of acetylcholine release and/or synthesis, cholinesterase desensitisation or adaptive changes in muscarinic, nicotinic or cholinesterase genes is thought to be deleterious for the concept of cholinergic replacement therapy in a chronic disorder such as Alzheimer's disease.

In this study ChE gene expression is upregulated with sub chronic metrifonate. One behavioural aspect of cholinergic activation, i.e. adverse reaction to cholinergic overstimulation becomes hyposensitive. However, the other, cognitive improvement is generally spared. It is not known as to why this is the case, however the increase in gene expression is only two fold and it is possible that other mechanisms within the brain are responsible for maintaining this cognitive improvement.

Effects of Metrifonate in the Passive Avoidance Task in Rats.

Abstract

The aim of the experiments described was to assess the effects of acute and sub-chronic cholinesterase inhibition on scopolamine induced amnesia. Acute metrifonate had no effect on the performance deficits during the retention session. When given sub-chronically before testing metrifonate effectively inhibited the scopolamine-induced deficit.

Although this cholinesterase inhibitor was able to antagonise scopolamine- induced deficits in the passive avoidance task it is necessary to confirm the effects of compounds in other tasks such as the Morris water escape task. The passive avoidance task is a fast way to assess the effects of putative cognition enhancers. However it is relatively complex and poorly understood and therefore can be taken as a first indication only that a compound might possess cognition enhancing properties.

Introduction

The passive or inhibitory avoidance test is considered to be a useful early screening test to identify putative cognition enhancing compounds in rodents. Animals with impaired performance are needed to test putative cognition enhancers. Administration of scopolamine (Imanishi *et al*, 1997) is a common method to induce amnesia of the aversive event, i.e. the footshock in the passive avoidance task. Compounds are usually tested in scopolamine-treated rats.

The scopolamine model of ageing and dementia assumes that the resulting behavioural impairments reflect the cholinergic dysfunction seen in AD (Wesnes et al, 1991). This study investigated the effects of metrifonate presently being studied or used for AD treatment. Metrifonate has also been shown to alleviate scopolamine-induced deficits in rats (Itoh, *et al*, 1997: Riekkinen *et al*, 1996).

Previous studies of ChEI's in the passive avoidance task report on the acute effects of metrifonate (Itoh, *et al*, 1997; Riekkinen *et al*, 1996). However, putative therapeutic agents for chronic ailments are likely needed to be administered over a prolonged period. Therefore the second aim of the study was to determine the effects of a sub-chronically administered ChEI on the passive avoidance task using metrifonate and to compare this with the acute effects of these compounds

4.1: Effects of acute metrifonate in the passive avoidance task

Animals: A group of 40 male HsdCpd: Wu (Harlan Wistar) rats (supplied by Harlan-Winkelmann, Borchen) were used. They were approximately 10 weeks old at the beginning of the experiment. Their weights ranged from 220-240 grams. The rats were housed in pairs in standard Mackrolon™ type III cages in which food and water was continuously available, with a 12:12 hour light/ dark cycle, lights on at 07.00 am.

Drug Administration: Rats (N = 8) were treated with 30, or 60 100 mg/kg Metrifonate. The compound was administered per os (p.o.) 30 minutes prior to the start of the shock trial in an application volume of 5 ml/kg. Controls received the vehicle (Sodium citrate buffer, pH 5.5). Scopolamine (1 mg/kg was administered 30 minutes prior to the shock session, interperitoneally (i.p.) in an application volume of 2 ml/kg, i.e. scopolamine was administered in conjunction with metrifonate before the start of the experiment. Control groups are vehicle alone and scopolamine with vehicle.

Apparatus: The inhibitory apparatus consisted of a two-compartment box with a light compartment and a dark compartment, each measuring 36 (height) * 27 (depth) * 37 (width) cm. The apparatus was made of black plastic, except for the sidewalls of the light compartment, which were white. The floor consisted of a metal grid connected to a shock scrambler.

A guillotine door that could be raised 9 cm separated the two compartments. A threshold of 2 cm separated the two compartments when the guillotine was raised. When the door was open, the illumination in the dark compartment was about 2 lux. The light intensity was about 500 lux at the centre of the light compartment. The equipment was placed in a noise box, situated in a room illuminated by red fluorescent strip lights.

Method: Two habituation sessions, one shock session and a retention session were given, separated by an inter-session interval of 24 hours (see figure 1). In the habituation sessions and the retention session the rat was allowed to explore the apparatus for 300 seconds. The rat was placed in the light compartment, facing the wall opposite to the guillotine door. After an accommodation period of 15 seconds, the guillotine door was opened so that all parts of the apparatus could be visited freely. In the shock session the guillotine door between compartments was lowered as soon as the rat had entered the compartment with its four paws, and then a 1 mA shock for 2 sec was administered. The rat was removed from the apparatus 10 seconds after shock termination and put back in its home cage. The procedure during the retention session was identical to that of the habituation sessions.

Analysis: Two variables were selected for further analyses:

1. The stepthrough latency, that is the first latency of entering the dark compartment , and
2. The time spent in the light compartment.

Whenever a rat did not enter the dark compartment, variables *1* and *2* (above) were ascribed a value of 300 seconds. Differences between groups on these variables were analysed for each session by analysis of variance (ANOVA), complemented with Fisher's LSD post hoc comparisons.

In this chapter '*antagonism*' refers to a full reversal of the scopolamine effect and that there is no significant difference between the vehicle and dose group. '*Partial antagonism*' refers to a reversal of the scopolamine effect but there is still some significant difference between the vehicle and dose group.

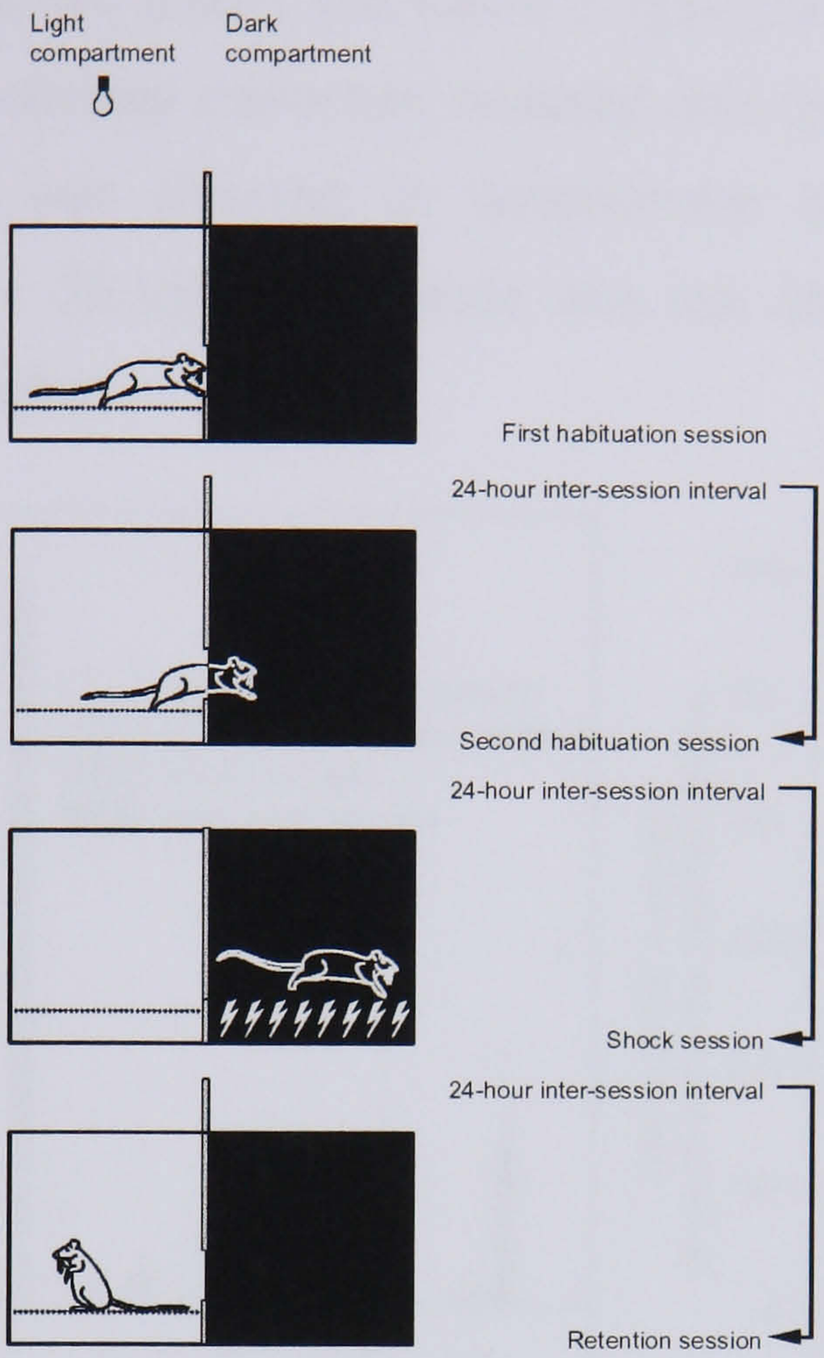


Figure 1: Schematical diagram of the procedure of the passive avoidance task. Each of the four sessions was separated by a 24hr inter-session interval.

Results

Acute metrifonate, (see figure 2)

The five treatment groups had small differences in the latency to enter the dark compartment during the first ($F_{4,36}$ = 3.18, $p < 0.05$) habituation session. No differences were observed for the latency to enter the dark compartment in the second habituation session ($F_{4,36}$ = 1.49, n.s) and the shock session ($F_{4,36}$ = 1.96, n.s), see figure 2, *left panel*). The treatments affected the latencies in the retention session ($F_{4,36}$ = 17.85 $p < 0.001$). Fisher's LSD post hoc analysis with a Bonferroni correction revealed that performance during the retention session was affected by scopolamine treatment (Vehicle vs Scopolamine: 273.75 ± 29.16). Metrifonate was not able to antagonise the scopolamine –induced deficit.

The total time spent in the light during the retention session differed between the groups ($F_{4,36}$= 9.19, p< 0.001, see figure 2 *right panel).* Fisher's LSD post hoc analysis with a Bonferroni correction revealed that the performance during the retention session was affected by scopolamine treatment (Vehicle vs Scopolamine: 166.87 ± 36.16). Metrifonate was not able to antagonise the scopolamine-induced deficit.

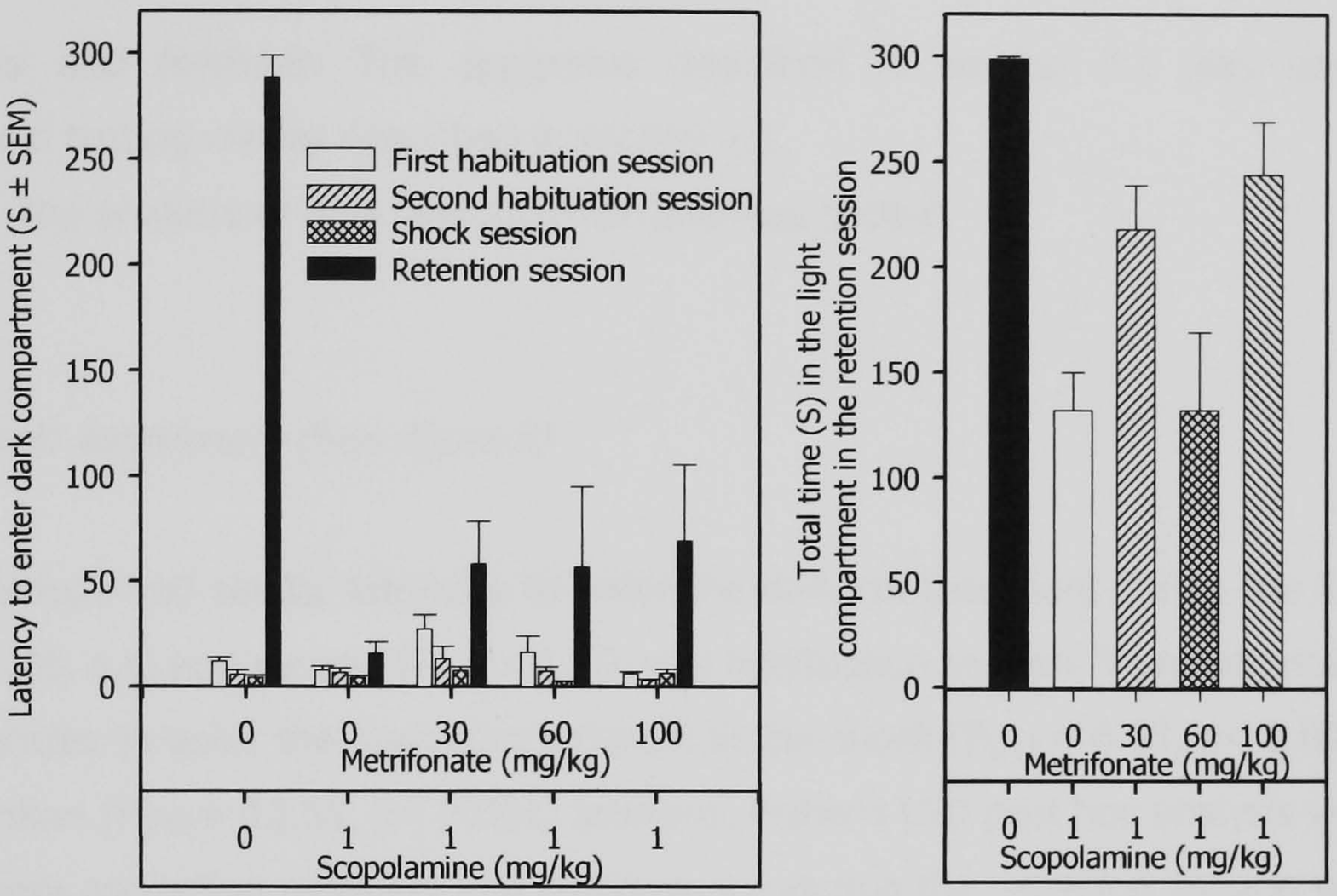


Figure 2: Effects of acute metrifonate during scopolamine induced amnesia on the performance of rats in the passive avoidance task. Data is depicted as means (N=8) and standard errors of the means (SEM) to enter the dark compartment during the first and second habituation session, the shock session and the retention session (*left panel*) and the total time spent in the light compartment during the retention session (*right Panel*).

4.2: Effects of sub-chronic metrifonate in the passive avoidance task

Animals: One group of male HsdCpb: WU (Harlan Wistar) rats (supplied by Harlan- Winkelmann, Borchen) were used. They were approximately 4 months old at the beginning of the experiment. Their weights ranged from 380-420 grams. The rats were housed in groups of two in standard Makrolon™ type III cages in which food and water was continuously available, with a 12:12 hour light/ dark cycle, lights on at 07.00 am.

Drug Administration: Rats (N= 8) were pre-treated once daily with 10, 30, 60 or 100 mg/kg Metrifonate in a volume of 5 ml/kg for 10 weeks in order to induce steady state cholinesterase inhibition. Controls received the vehicle (sodium citrate buffer, pH 5.5) under identical conditions. During training this treatment schedule was continued. Animals were

applicated per os (p.o.) 30 minutes prior to the start of the trials in an application volume of 5 ml/kg. Scopolamine (1 mg/kg) was administered 30 minutes prior to the shock session, interperitoneal (i.p.) in an application volume of 2 ml/kg. Scopolamine was administered in conjunction with metrifonate before the start of the experiment.

Apparatus and Methods: The apparatus described in section 2.1 was used. Behavioural testing was as described in section 2.1

Analysis: The analysis of data was as in the previous section

Results

Sub-chronic metrifonate (See figure 5)

The six groups had similar latencies to enter the dark compartment during the first ($F_{5,42}$ = 1.28, n.s) and second ($F_{5,34}$ = 1.25, n.s) habituation session. They differed in their latencies to enter the dark compartment in the shock ($F_{5,42}$ = 6.63, $p < 0.001$) and retention ($F_{5,42}$ = 12.53, $p < 0.001$) sessions. Fisher's LSD post hoc analysis with a Bonferroni correction revealed that performance during the retention session was impaired by scopolamine treatment (Vehicle vs Scopolamine: 294.5 ± 53.601). This effect was partially antagonised by 60mg/kg metrifonate (Scopolamine vs 60 mg/kg metrifonate: 150.75 ± 46.42).

The total time spent in the light ($F_{5,34}$ = 13.81, $p < 0.001$, see figure 5 *right panel*) during the retention session differed between the groups. Post hoc analysis with a Bonferroni correction revealed that, the total time spent in the light was decreased by scopolamine treatment (Vehicle vs Scopolamine: 230.0 ± 45.3) and this decrease was partially antagonised by 10 mg/kg (144.13 ± 39.43) and 60 mg/kg metrifonate (176.25 ± 39.43).

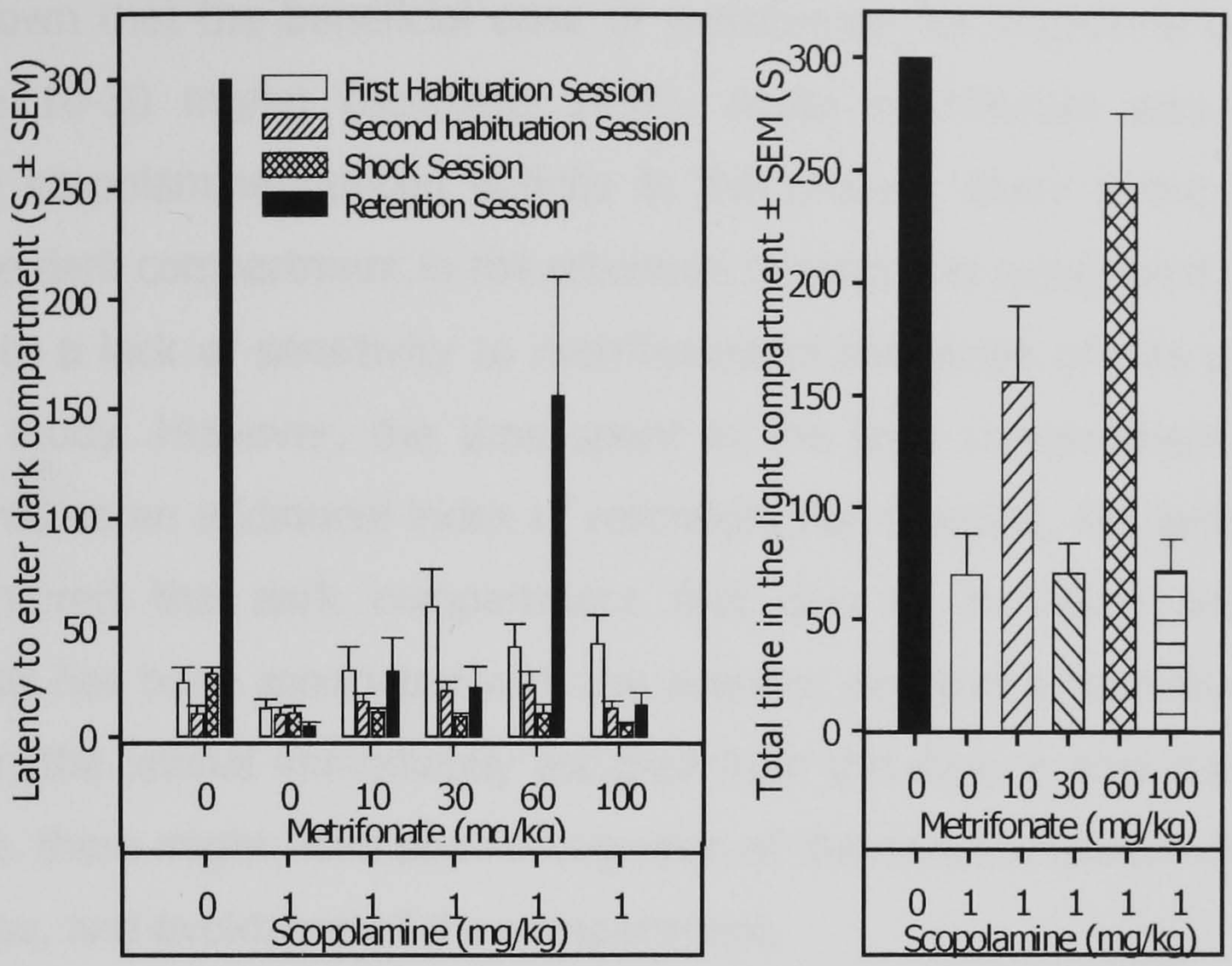


Figure 5: Effects of sub chronic metrifonate on scopolamine induced amnesia in rats in the passive avoidance task. Data is depicted as means (N=8) and standard errors of the means (SEM) to enter the dark compartment during the first and second habituation session, the shock session and the retention session. (*left panel*) and the total time spent in the light compartment during the retention session (*right Panel*).

Discussion

Summary of results

Scopolamine interferes with learning and memory in humans (Beatty et al., 1986) and experimental animals (Stevens, 1981; Sutherland, 1982) by blocking muscarinic receptors in the basal forebrain area. The present study compared the effects of acute and sub-chronic administration of the cholinesterase inhibitors metrifonate, donepezil and rivastigmine against scopolamine induced memory impairments in the passive avoidance task.

Acute metrifonate studies in rats, using the passive avoidance paradigm, revealed that this compound alleviated scopolamine-induced deficits (Riekkinen *et al* 1996; Itoh, *et al*, 1997).

Metrifonate also facilitated passive avoidance retention in 23-month-old and 27-month-old rats (Riekkinen *et al*, 1996). Previous studies with metrifonate have shown that the beneficial dose of metrifonate for improving cognition is between 10-30 mg/kg (Schmidt, 1997). Acute metrifonate was unable to alleviate scopolamine-induced deficits in the present study if the latency to enter the dark compartment in the retention session was considered. This could be due to a lack of sensitivity to metrifonate of the strain of rats used in the present study. However, the time spent in the light compartment might be considered as an additional index of retention. For example, the animals might have entered the dark compartment fast due to increased arousal (the apparatus has been associated with the aversive event of a foot shock). Then, however, the animal immediately escaped from the dark to stay mostly in the light. I.e. there might have been recognition of the dark compartment, followed by escape, and avoidance of the compartment.

In the second experiment the effects of a sub-chronically administered ChEI on the passive avoidance task using metrifonate treatment was determined. The compound was well tolerated, in that no adverse side effects were observed by the animals. Sub-chronic metrifonate (60 mg/kg) ameliorated the scopolamine-induced amnesia during the retention session.

Repeated administration of metrifonate to rats or rabbits resulted in accumulation of the drug resulting in plasma steady state levels, decreasing the peaks and troughs of inhibition observed after a single dose. Continuous peak inhibition was achieved after 15-20 single doses (Kronforst et al, 1997). Compared with acute administration adverse events are lower with repeated administration without affecting the cognition enhancing potency of the compound (Blockland et al, 1995). This present study revealed that metrifonate alleviated the scopolamine- induced deficit in animals that had received a once daily dose for 12 weeks, whereas an acute single dose of metrifonate was ineffective. Therefore it is a possibility that the long lasting inhibition of cholinesterase induced by metrifonate may contribute to its ameliorating effects on the impairment of learning and memory in the passive avoidance task.

The inability of the higher dose of subchronic metrifonate (100 mg/kg) to antagonise memory deficits induced by scopolamine may be related to excessive cholinergic stimulation in the central nervous system.

Problems with the P.A. task

Scopolamine, a muscarinic antagonist has been shown to impair behaviour in a variety of tasks (Buresova *et al*, 1986; Rush, 1988) and scopolamine treated animals have been used as a model of ageing and dementia since they show some of the cognitive impairments seen in AD (Wesnes et al, 1991). In the passive avoidance paradigm scopolamine induced decreases in retention latency (latency to re-enter, step through or step down) are considered to represent a measure of amnesia. However, although the effects of scopolamine on a variety of tasks are robust and reproducible there are differences between the scopolamine model and the symptoms of Alzheimer's disease. The impairments are induced by post-synaptic blockade rather than by pre-synaptic destruction of cholinergic neurons, whereas in the case of AD this is largely a pre-synaptic degenerative phenomenon. Also, with scopolamine, receptor blockade is widespread throughout all areas of the brain and this contrasts with a pattern of more specific areas that appear to be affected in brains of AD patients (Decker, 1995; Blokland, 1995).

In the passive avoidance task the use of electric shocks may not only have affects on mnemonic processes but also produce on stress, anxiety emotionality, changes in motivation, and in arousal levels etc (van Dijken, 1992). Previous studies have shown that a particular dose of scopolamine interacts with different levels of shock, producing decreases in retest response latencies only at the lowest footshock intensities tested (3 and 4 mA) but not at higher intensities (4.1- 5 mA; Cruz- Morales et al, 1990). It has also been shown that stress attenuates the effects of

scopolamine in a passive avoidance task. One theory suggests that scopolamine spares the memory for the shock punishment and alters re-entry latencies by alternative mechanisms (Calhoun et al, 1968). The possible impact of shock motivated tasks on other aspects of behavioural, such as stress should be kept in mind.

There is growing agreement that the validity of the passive avoidance task is low and that this test yields a high number of false positive hits. A number of compounds that have been listed active in the passive avoidance task and have been tested clinically For example; Haloperidol (Devanand et al, 1989), choline (Becker and Giacobini, 1988), pyridostigmine (Becker and Giacobini, 1988), 4-aminopyridine (Davidson et al, 1988) and nicotine (Newhouse et al, 1988) have failed to produce any significant therapeutic effect.

Conclusions

Different ChE inhibitors are able to antagonise, at least partially, scopolamine induced performance deficits in the retention session of the PA task. This holds true after acute and chronic treatment with the ChE inhibitors. Though simple and fast, the passive avoidance task is complex and poorly understood. A positive effect in this task does not necessarily indicate that a substance is a cognitive enhancer. Instead, the conclusion should be that the drug affects behaviour in one way or another, possibly by modulation of CNS processes (van der Staay, 1998). A single test procedure based on a particular set of stimulus responses cannot be sufficient for determining a drug's effect on cognitive processes. Along with the limitations of the passive avoidance task, further verification, using more sophisticated tasks such as the Morris water escape task (Chapters 4-6) or timing behaviour (Chapter 8) is required before final conclusions can be drawn about the potential of drugs to improve cognitive performance.

Effects of Metrifonate in the Morris Water Maze Escape Task in Normal Rats and Mice

Abstract

The experiments described in this chapter were designed to assessed the effects of subchronic metrifonate, a second generation ChEI currently available. The effects of this compound were assessed in neurologically normal rats in the standard Morris maze water escape task. Also studied were the effects of acute and sub-chronic administration of metrifonate in the wild type C57/BL mouse.

Metrifonate did not to improve spatial learning at any dose tested, in neurologically normal rats.

C57/BL mice performed well in the Morris water maze while the administration of metrifonate did not improve the spatial reference memory performance of these neurologically normal mice. Sub-chronic administration of 30 mg/kg metrifonate had beneficial effects in mice. They escaped to the platform faster than the vehicle treated control group.

Introduction

Alzheimer's disease (AD), the most common dementia in the elderly causes severe cognitive dysfunction. One of the deficits observed in patients suffering from AD is an impairment in visuospatial discrimination (Adams et al, 1997). Spatial deficits are not exclusively restricted to humans: aged rats often show an impaired performance in spatial learning tasks (Barnes, 1988). One such task used to measure spatial discrimination in rodents is the Morris maze water escape task (MWM; see chapter 1).

The strength of the MWM is the availability of procedures for evaluating the strategy being used by the animal to locate the platform, and for dissociating pharmacological impairments of memory processes from non-mnemonic deficits.

An animal can use three different strategies to reach the platform during a swimming trial (Brandeis, 1989). It can use a learned sequence of movements, which brings it to the platform (*praxis* strategy); it can approach the platform using proximal cues (*taxis* strategy); or it can navigate to the platform using information about the platform's location within spatial configuration of distal cues (*mapping* or *spatial* strategy). An animal may conceivably use both praxis and mapping strategies to locate the hidden platform when random starting positions are applied (Dalm, 2000).

Standard performance measures during the acquisition phase of the MWM task usually include escape latency (time required to reach the platform and length of the swimming path (distance travelled). Lindner (1997) has suggested that path length might be the most appropriate index of cognitive performance in the MWM. Learning and cognitive performance can be assessed simultaneously and the swim speed can be used to assess motoric and motivational deficits within each learning trial. After a series of acquisition trial blocks, a probe trial is usually performed in which the rat is permitted to swim freely about the pool without any platform present. The idea is that if the rat spends more time in the quadrant that previously contained the platform or crosses over the old platform position a small region around the previous platform position (annulus) more often than in equivalent areas in the other three quadrants. In this study a time of 30 seconds was used, as this was the time the animal was required to stay on the platform during the acquisition trials. These measures quantify the strength and accuracy of the original learning (Brandeis *et al*, 1989). Other advantages of this task are: no extensive pre- training is required, and normal animals learn where the platform is located very quickly, so testing can be carried out over a short period of time, usually within one week.

ChE inhibitors have been shown to be effective in ameliorating learning and memory in animals with experimentally induced cognitive impairments (e.g. medial septal lesions; Riekkinen et al, 1990; basal forebrain lesions; Niigawa et al, 1995; Rogers *et al*, 1991). However, the validity of these animals as models for AD or for specific symptoms of the disease has been questioned (Fibiger, 1991; Dunnett et al, 1991). The aim of the present study was to assess the effects of metrifonate used in the treatment of AD in normal animals, which are young animals with no

pharmacologically or mechanically induced impairments. Secondly, previous studies with metrifonate (van der Staay *et al*, 1996b) investigated the effects of acute administration of metrifonate (12.5mg/kg, p.o.), administered before each of the daily acquisition sessions. The compound facilitated the acquisition of the Morris water escape task; metrifonate-treated rats swam a shorter distance to reach the escape platform than did the vehicle-treated rats. Here, we extended previous work to study the effects of a sub-chronically administered metrifonate in the MWM paradigm in the rat.

Finally, a commonly used species for Alzheimer's transgenic manipulations is the C57BL mouse. However few studies have assesses the effects of ChEI's of the wild type of this mouse strain on learning and memory. Therefore, we studied the effect of acute and sub-chronic metrifonate in neurologically intact C57 BL mice to determine as to the innate sensitivity to the cognition-enhancing properties of metrifonate.

5.1: Effects of Sub-Chronic Metrifonate in the Morris Maze Water Escape in Normal Harlan Wistar Rats

Procedure

Animals: One group of 40 male HsdCpb: WU (Harlan Wistar) rats (supplied by Harlan-Winkelmann, Borchen, Germany) were used. They were approximately 10 weeks old at the beginning of the experiment. Their weights ranged from 220 to 250 grams. The rats were housed in groups of two in standard Makrolon™ type III cages in which food and water was continuously available, with a 12:12 hour light/ dark cycle, lights on at 07.00 am.

Drug Administration: Rats (N= 8-10) were pre-treated orally once daily with 10, 30, 60 or 100 mg/kg Metrifonate (see experimental protocol 1, Appendix 1), in an application volume of 5 ml/kg for 3-5 weeks in order to induce steady state cholinesterase inhibition. Controls received the vehicle (Sodium citrate buffer, pH 5.5) under identical conditions. During training this treatment schedule was continued. Vehicle or test compounds were administered 30 minutes prior to the start of the training sessions.

Apparatus and Methods: Morris water escape performance was assessed in a water tank which consisted of a circular grey tub with a slightly sloping wall (Material: polyethylene; inner dimensions: diameter at top 153 cm, diameter at bottom 143 cm, depth 63 cm), filled with 43.5 cm of clear tap water at a temperature of approximately 22°C. The escape platform consisted of a grey polyethylene cylinder (diameter 10.8 cm), submerged 1.5 cm below the surface of the water. In this version of the test the water was not made opaque because the grey escape platform was virtually invisible in the grey tank. The water tank was situated in a room illuminated by white fluorescent tubes. Abundant extra-maze cues were provided by the furniture in the room, including desks, computer equipment, the presence of the experimenter, and by a radio on a shelf that was playing softly (*See Figure 1*). All testing was done between 9:00 and 15:00. A video camera, mounted in the centre above the circular pool, provided a picture of the pool on a TV-monitor.

The movements of the rat were registered automatically by a video-tracking system (EthoVision®, Noldus Information Technology,
Wageningen, The Netherlands) and stored in an MS-DOS compatible microcomputer.

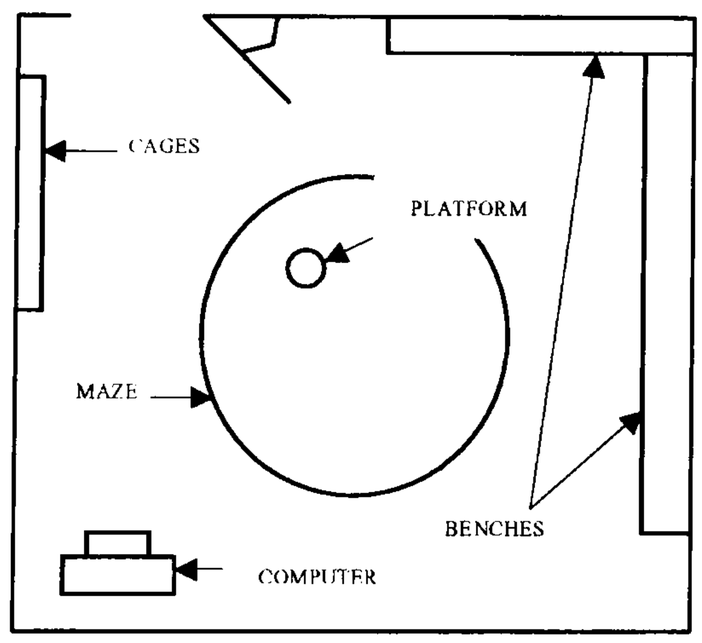


Figure 1: Experimental Room Layout

The rats were tested in the Morris water escape task with four trials per session on five successive days (Morris, 1984) The test compound or vehicle was injected intraperiteoneally, 30 minutes before each daily training session. A trial was started by placing a rat into the pool, facing the wall of the tank. Each of the four starting positions (arbitrarily assigned north, south, east, west) was used in a series of four trials; their order was randomised. The escape platform was always in the same

fixed position in the west quadrant. A trial was terminated as soon as the rat had climbed onto the escape platform or when 90 seconds had elapsed, whichever event occurred first (*see figure 2*). Each rat was allowed to stay on the platform for 30 seconds. Then it was taken from the platform and the next trial started immediately. Rats that did not find the platform within the 90 seconds were put on the platform by the experimenter and were allowed to stay there for 30 seconds.

After the fourth trial on the fifth session, an additional trial was given as a probe trial: the platform was removed, and the time spent in the four quadrants was measured for 30 seconds. The area was around the platform is defined as the annulus region. On the probe trial, all rats started from the same position, opposite to the quadrant where the escape platform had been positioned during acquisition.

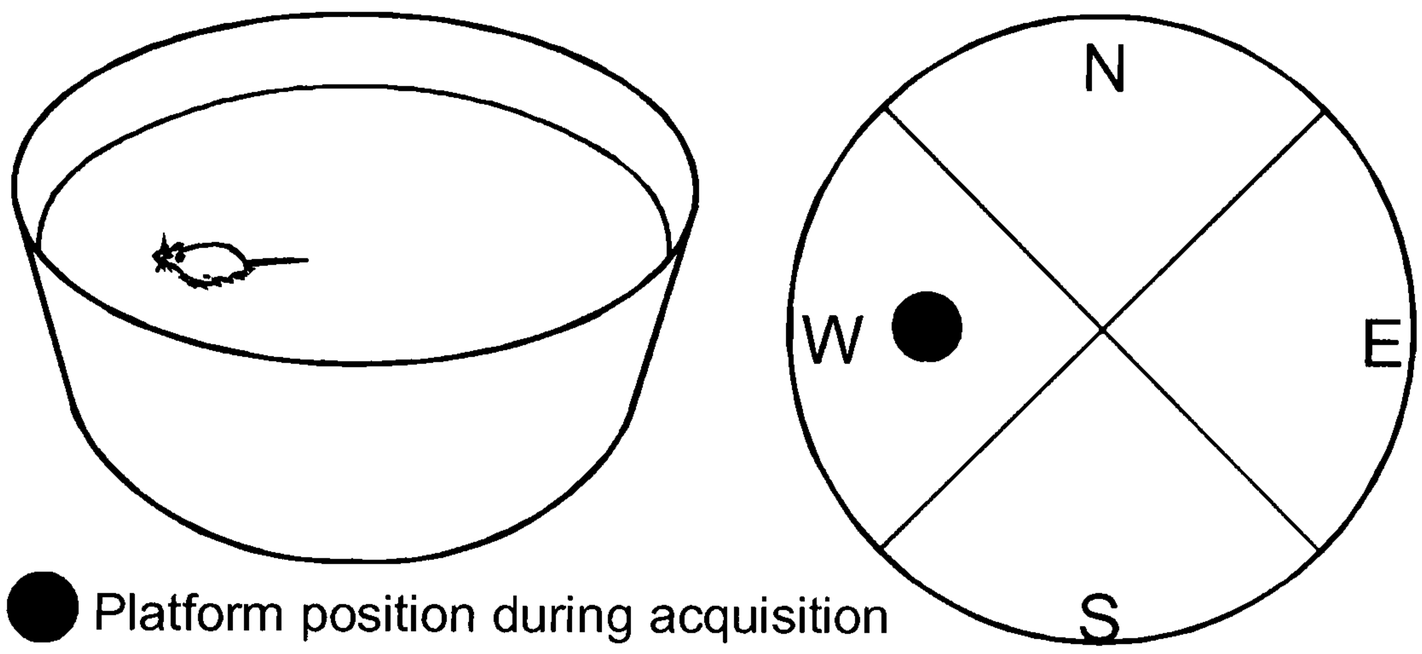


Figure 2. The Standard Morris Maze. The invisible submerged platform was always in the same quadrant. A trial was terminated as soon as the rat had swum and climbed on to the platform, as depicted on the illustration.

Analysis: EthoVision™ analysed the distance travelled, the distance to point (Platform), the time spent in zone and the velocity of the animal. The mean per animal, per trial was then calculated

This data was then analysed per animal per session by the SAS GLM-procedure. The following three parameters for acquisition of the water escape task were analysed statistically: the escape latency (in s); the distance travelled (in cm); and the swimming speed (in $cm*s^{-1}$). They were assessed with a TREATMENT*SESSION analysis of variance (ANOVA) with repeated measures over SESSIONS. Analysis was

supplemented by T- test post hoc comparisons. A difference between groups was considered significant if the p value was below 0.05. Treatment effects on the time spent in the annulus during the probe trial was assessed by ANOVA. Drug effects on the time and distance travelled in the quadrant was assessed with a repeated measures ANOVA over QUADRANTS. In addition, treatment effects were further evaluated by T test post hoc comparisons ($p< 0.05$).

Results

Metrifonate

The data obtained during the acquisition of the experiment is summarised in fig. 3. The performance during the probe trial is depicted in fig. 4

Acquisition Trials

Escape latency: All rats started from the same level of performance (First session: $F_{4,36}$ = 0.28, n.s, see figure 3, *upper left panel*). Averaged over all sessions, Metrifonate had no influence on the escape latency (GENERAL MEAN: $F_{4,35}$ = 0.55, n.s). The escape latencies of the groups decreased in the course of training (SESSIONS:$F_{4,140}$ = 48.43, $p<0.01$), however this rate of learning was not affected by metrifonate treatment (SESSIONS by TREATMENT: $F_{16,140}$ = 0.71, n.s).

Quadrant Entries: The rats all started from the same level of quadrant entries (First Session: $F_{4,35}$ = 0.20, n.s see figure 2, *upper right panel*). Overall, the treatment of metrifonate did not influence the mean number of quadrants entered during swimming (GENERAL MEAN: $F_{4,35}$ = 0.62, n.s.). During training the rats reduced their quadrant entries (SESSIONS: $F_{4,140}$= 31.75, $p<0.01$). The rate of improvement across the sessions was similar for all groups (SESSIONS by TREATMENT: $F_{16,140}$ = 0.37, n.s).

Distance Travelled: The path lengths to escape onto the platform were all similar at the start of training (First Session: $F_{4,35}$ = 0.37, n.s see figure 3, *lower left panel*). Averaged over all sessions, metrifonate did not decrease the mean distance travelled during training (GENERAL MEAN: $F_{4,35}$= 0.44, n.s.,). All groups decreased the distance swum across the sessions (SESSIONS: $F_{4,140}$= 32.36, $p<0.01$).

The distance travelled across the sessions was similar for all treatment groups (SESSIONS by TREATMENT: $F_{1,140} = 0.60$, n.s), i.e. there was no effect of treatment with metrifonate.

Swimming Speed: All rats started from the same level of performance (First Session: $F_{4,35} = 1.10$, n.s). Averaged over all sessions, Metrifonate had no influence on the swimming speed (GENERAL MEAN: $F_{4,35} = 0.38$, n.s., see figure 2, Lower right panel). The swimming speed of the rats changed in the course of training (SESSIONS:$F_{4,140} = 10.63$, $p<0.01$), this was affected by metrifonate treatment (SESSIONS by TREATMENT: $F_{16,140} = 1.89$, $p<0.05$). However further analysis by Duncan's multiple range test did not clarify the nature of this difference between treatment groups.

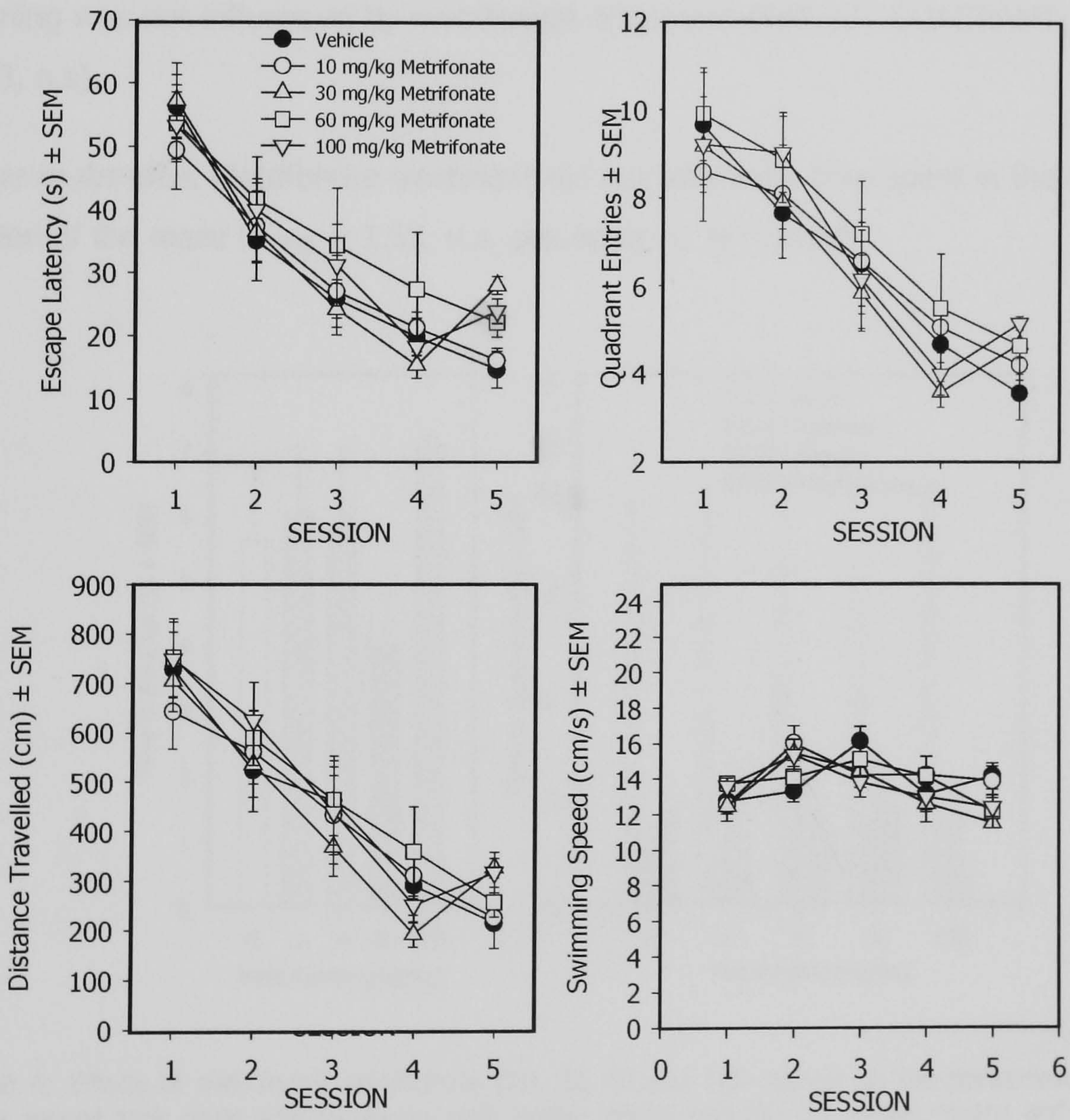


Figure 3: Effect of sub-chronic metrifonate (10, 30, 60 and 100 mg/kg) on the performance in the water escape task by young male Harlan Wistar rats (N= 8 per group). Session means and standard errors of the means (SEM) are depicted for latencies (s) to escape onto a platform (upper left panel), number of quadrant entries (upper right panel), distance travelled (cm; lower left panel) and swimming speed ($cm*s^{-1}$; lower right panel).

Probe Trial.

Time in Quadrant: The time the groups spent in the four quadrants were different (QUADRANTS: $F_{3,105}$ = 158.19, $p<0.001$; see figure 4, right panel) and this difference was affected by metrifonate treatment (QUADRANTS by TREATMENT: $F_{12,105}$ = 1.94, $p<0.05$;). However the bias for the quadrant in which the platform had been positioned during training was not influenced by metrifonate treatment (TARGET QUADRANT: $F_{4,35}$ = 1.04, n.s).

Distance Travelled: The distance travelled in the four quadrants was different (QUADRANTS: $F_{3,105}$ = 156.30, $p<0.001$; Data not shown). This difference was not affected by metrifonate treatment (QUADRANTS by TREATMENT: $F_{12,105}$ = 1.42, n.s), and the bias for the quadrant in which the platform had been positioned during training was not influenced by metrifonate treatment (TARGET QUADRANT: $F_{4,35}$ = 0.63, n.s).

Time in Annulus: Metrifonate treatment did not affect the time spent in the annulus region of the maze ($F_{4,35}$ = 1.53, n.s; see figure 4, *left panel*)

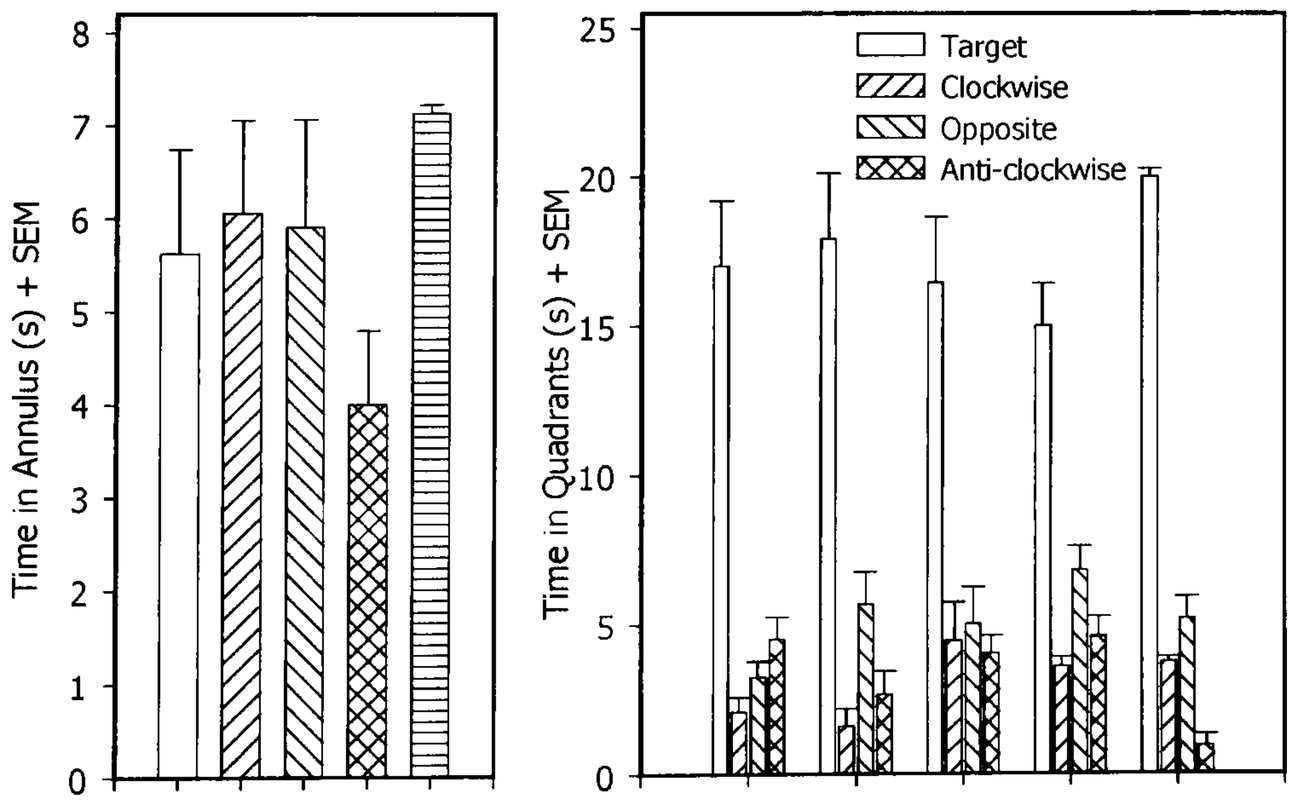


Figure 4: Effects of sub-chronic metrifonate (10, 30, 60 and 100 mg/kg) on the performance in the water escape task probe trial by young male Harlan Wistar rats (N= 8). Group means and standard errors of the means (SEM) are depicted for Time spent in the Annulus (s) (*left panel*) and time spent in the quadrant (*right panel*)

5.2: Effects of Acute Metrifonate In Normal C57BL 6J Mice

Animals: Male C57BL/ 6J mice, aged about 10 weeks, weighing approximately 20-25 grams, were supplied by Winkelmann (Borchen, Germany). The animals were housed in groups of ten in standard Makrolon™ type III cages. They were kept under an artificial 12-hour light/12 hour dark regimen (lights on from 7.00 to 19.00) in a temperature (ca. 21.5°C) and humidity (50%) controlled animal room. Water and food were available ad libitum. Before testing the animals were transferred to the experimental room where they were housed for the duration of the testing period. Housing conditions were similar to those in the animal room.

Apparatus: The water tank used was a circular grey tank (polyethylene) with a slightly sloping wall (inner dimensions: diameter at top 74cm, diameter at bottom 66 cm, depth 54 cm.) The tank was filled with clear tap water at a temperature of approximately 22°C. The escape platform was a grey polyethylene cylinder (7.3 cm width) submerged 0.6 cm below the surface of the water.

The tank was situated in a room illuminated by white fluorescent tubes, (the lights directly above the maze were turned off to prevent reflection). Blinds were closed to prevent the entrance of natural light. Extra maze cues were provided by the furniture in the room, including desks, computer equipment, the presence of the experimenter and by a radio on a shelf that was playing softly. All testing was done between 9.00 and 14.00

Methods: The same procedure as described in section 2.1 was followed.

Drug administration: Mice (N= 8 per group) were treated each day of training with 3, 10 or 30mg/kg Metrifonate. Controls, which received the vehicle (Sodium citrate buffer, pH 5.5). Drug and vehicle was applicated per os (p.o.) 30 minutes prior to the start of the daily training sessions in an application volume of 20 ml/kg.

Analysis: The data analysed were as explained in section 2.1. Two animals were removed from analysis due to visible side effects.

Results

Acquisition Trials (see figure 5)

Escape latency: All groups of mice started from the same level of performance (First session: $F_{3,26} = 0.76$, n.s). Averaged over all sessions, Metrifonate had no influence on the escape latency ($F_{3,26} = 0.99$, n.s). The escape latencies of the mice decreased in the course of training ($F_{4,104} = 36.07$, $p<0.01$), however the rate of learning was not affected by metrifonate treatment ($F_{12,104} = 0.60$, n.s).

Quadrant Entries: The treatment groups started from the same level of quadrant entries (First Session: $F_{3,26} = 0.92$, n.s). The treatment with metrifonate did not influence the mean number of quadrants entered during swimming (GENERAL MEAN: $F_{3,26} = 2.83$, n.s., see figure 6, *upper right panel*). During training the mice reduced their quadrant entries (SESSIONS: $F_{4,104} = 29.61$, $p<0.01$) but the rate of improvement across the sessions, however, was similar for all groups (SESSIONS by TREATMENT: $F_{12,104} = 0.63$, n.s).

Distance Travelled: The path lengths to escape onto the platform were all similar at the start of training (First session: $F_{3,26} = 2.04$, n.s). Averaged over all sessions, all groups of animals travelled a similar distance (GENERAL MEAN: $F_{3,26} = 1.23$, n.s., see figure 6, *lower left panel*). All mice decreased the distance swum during the sessions (SESSIONS: $F_{4,104} = 32.35$, $p<0.01$). The distance travelled across the sessions was similar for all groups (SESSIONS by TREATMENT: $F_{12,104} = 1.87$, n.s).

Swimming Speed: All mice started from the same level of performance (First session: $F_{3,26} = 2.5$, n.s). Averaged over all sessions, metrifonate had no influence on the swimming speed (GENERAL MEAN: $F_{3,26} = 0.30$, n.s., see figure 6, *lower right panel*). The swimming speed of the mice changed in the course of training (SESSIONS:$F_{4,104} = 6.92$, $p<0.01$), this was affected by metrifonate treatment (SESSIONS by TREATMENT: $F_{12,104} = 2.44$, $p<0.05$). Post hoc analysis did not clarify the nature of this interaction, except that post hoc comparisons per session revealed during session 3 the swimming speed was reduced by 3, 10 and 30 mg/kg metrifonate compared to the speed of the vehicle treated mice.

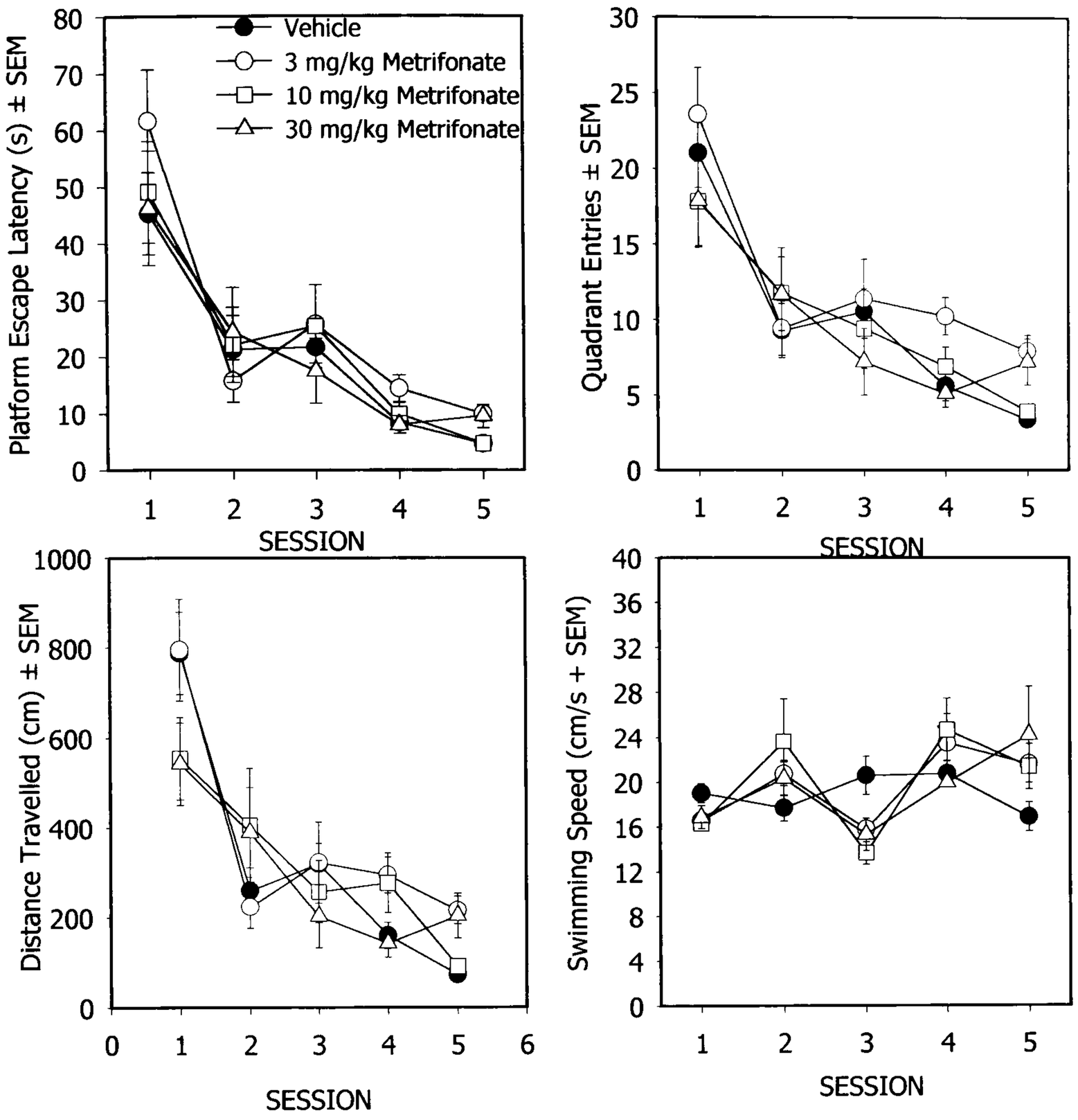


Figure 6: Effect of acute metrifonate (3, 10 and 30 mg/kg) on the performance in the water escape task by young male C57/BL mice (N= 8 per group). Session means and standard errors of the means (SEM) are depicted for latencies (s) to escape onto a platform (*upper left panel*), number of quadrant entries (*upper right panel*), distance travelled (cm; *lower left panel*) and swimming speed (cm/s*; lower right panel*).

Probe Trial (See figure 7).

Time in Quadrant: The time the groups spent in the four quadrants was different (QUADRANTS: $F_{3, 75} = 8.18$, $p<0.001$; see figure 7, *right panel*). This difference was not affected by metrifonate treatment (QUADRANTS by TREATMENT: $F_{9,75} = 1.39$, n.s.), and the bias for the quadrant in which the platform had been positioned during training was not influenced by metrifonate treatment (TARGET QUADRANT: $F_{3,26} = 0.94$, n.s).

Distance Travelled: The distance travelled in the four quadrants was different (QUADRANTS: $F_{3,75}$ = 8.24, p<0.001; d*ata not shown*) and this difference was affected by metrifonate treatment (QUADRANTS by TREATMENT: $F_{9,75}$ =2.63, p<0.05). However the bias for the quadrant in which the platform had been positioned during training was not influenced by metrifonate treatment (TARGET QUADRANT: $F_{3,26}$ = 1.24, n.s).

Time in Annulus: Metrifonate treatment did not affect the time spent in the annulus region of the maze ($F_{3,36}$= 1.03, n.s; see figure 6, *left panel*)

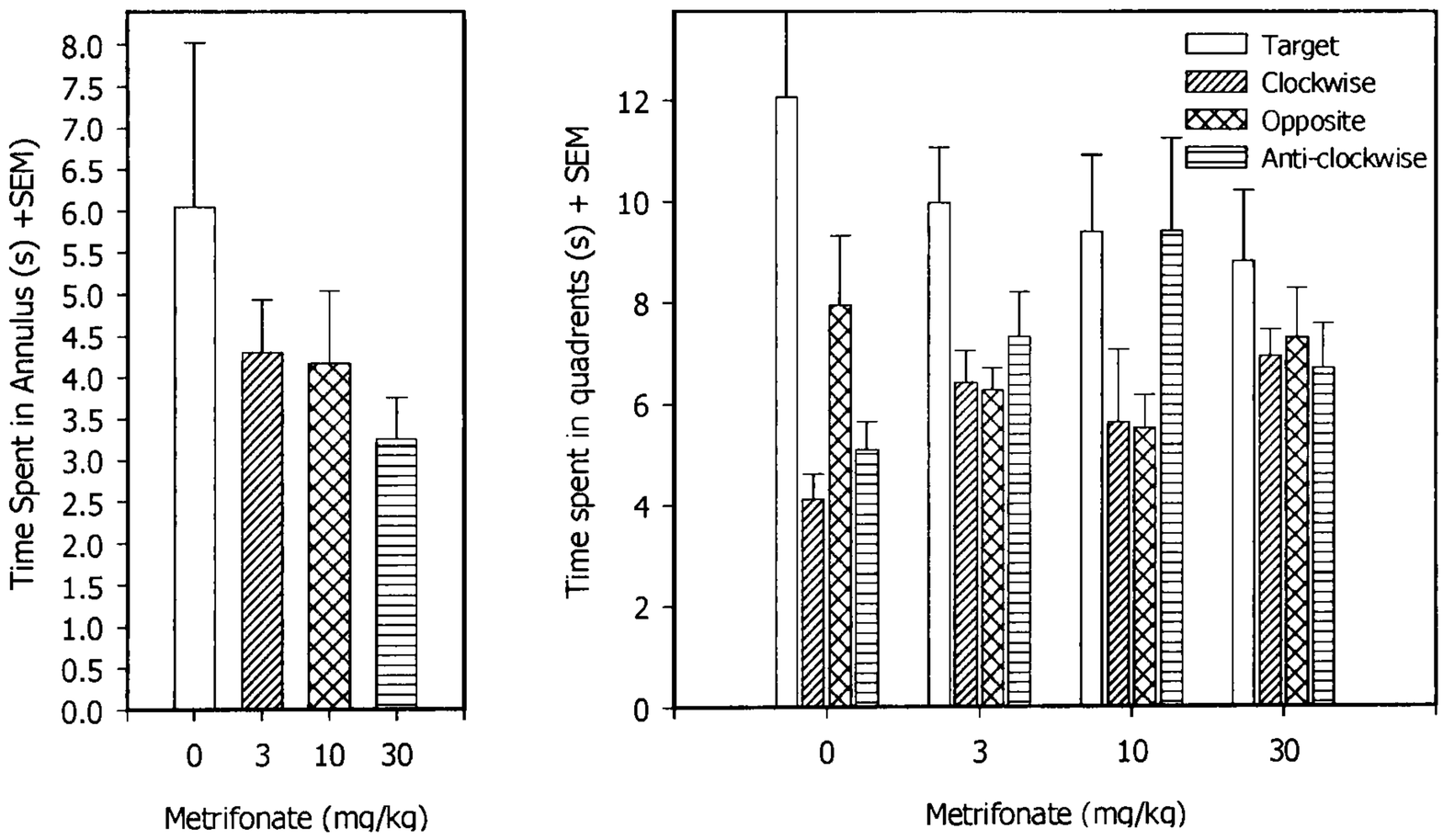


Figure 6: Effects of acute metrifonate (3, 10 and 30 mg/kg) on the performance in the water escape task probe trial by young male C57/BL Mice (N= 8 per group). Session means and standard errors of the means (SEM) are depicted for Time spent in the annulus (s) (*left panel*) and time spent in the quadrants (*right panel*).

5.3: Sub chronic Metrifonate in Normal C57BL 6J Mice in the Morris Maze

Animals: 40 Male C57/BL/6J mice, aged about 10 weeks, weighing approximately 18-25 grams, were supplied by Winkelmann (Borchen, Germany). The animals were housed in groups of ten in standard Makrolon™ type III cages. They were kept under an artificial 12

hour light/12 hour dark regimen (lights on from 7.00 to 19.00) in a temperature (ca.21.5°C) and humidity (50%) controlled animal room. Water and food were available ad libitum. Before testing the animals were transferred to the experimental room where the were housed for the duration of the testing period. Housing conditions were similar to those in the animal room.

Drug Administration: Mice (N= 10 per group) were pre-treated orally once daily with 10, 30, 60 mg/kg Metrifonate in an application volume of 20 ml/kg for 3 weeks in order to induce steady state cholinesterase inhibition. Controls received the vehicle (sodium citrate buffer, pH 5.5) under identical conditions. During training this treatment schedule was continued. Drug or vehicle was administered 30 minutes prior to the start of the training sessions.

Apparatus and Methods: The same procedure as described in section 5.2 was followed

Analysis: The same analysis as explained in section 5.1 was performed.

RESULTS

Acquisition Trials

Escape latency: All mice started from the same level of performance (First session: $F_{3,36}$ = 0.33, n.s). Averaged over all sessions, Metrifonate affected the escape latency (GENERAL MEAN: $F_{3,36}$ = 6.91, $p<0.01$, see figure 7 *upper left panel*). The escape latencies of the mice decreased in the course of training (SESSIONS: $F_{4,144}$ = 28.93, $p<0.01$). However this rate of learning was not affected by metrifonate treatment ($F_{12,144}$ = 1.09, n.s). Post hoc analysis by Duncans Multiple range test on the general mean revealed that 30 mg/kg metrifonate, on average had a shorter escape latency than the vehicle control group and 60 mg/kg metrifonate on average had a longer escape latency compared to the vehicle treated controls.

Quadrant Entries: On day one of training there was no difference in the number of quadrant entries (First Session: $F_{3,36}$ = 0.32, n.s). Metrifonate treatment did influence the mean number of quadrants entered (GENERAL MEAN: $F_{3,36}$ = 4.41, $p<0.01$., see figure 7, *upper right panel*). During training the mice reduced their quadrant entries (SESSIONS: $F_{4,144}$= 45.52, $p<0.01$). The rate of reduction of the number of quadrant entries across the sessions was not different between groups

(SESSIONS by TREATMENT: $F_{12,144}$ = 1.04, n.s). Post hoc analysis by Duncans Multiple range test on the general mean revealed that the 10 and 30 mg/kg dose had a lower number of quadrant entries than the vehicle control group.

Distance Travelled: The path lengths to escape onto the platform showed no difference at the start of training (First session: $F_{3,36}$ = 0.03, n.s). Averaged over all sessions, the groups had a different distance travelled during training (GENERAL MEAN: $F_{3,36}$ = 4.13, p<0.01., see figure 7, *lower left panel*). Post hoc analysis revealed that compared to vehicle, 10 and 30 mg/kg treated groups had a decreased path length. All groups of mice decreased the distance swum across the sessions (SESSIONS:$F_{4,144}$= 44.98, p<0.01). The decrease in distance travelled across the sessions was different for groups (SESSIONS by TREATMENT: $F_{12,144}$ = 6.80, <0.01). With the groups treated with 10 and 30 mg/kg having steeper learning curves.

Swimming Speed: On day one of training there was no difference in the swimming speed ($F_{3,36}$ = 1.28, n.s). Averaged over all sessions, Metrifonate showed no influence on the swimming speed (GENERAL MEAN: $F_{3,36}$ = 1.77, n.s., see figure 7, *lower right panel*). The swimming speed of the mice changed in the course of training (SESSIONS: $F_{4,144}$= 2.63, p<0.05), this was not affected by metrifonate treatment (SESSIONS by TREATMENT: $F_{12,144}$ = 1.17, n.s).

Probe Trial.

Time in Quadrants: The time the groups spent in the four quadrants were different (QUADRANTS: $F_{3,105}$ = 15.66, p<0.001; see figure 8, *right panel*). However, this difference was not affected by metrifonate treatment (QUADRANTS by TREATMENT: $F_{9,105}$ =0.47, n.s.). The bias for the quadrant in which the platform had been positioned during training was not influenced by metrifonate treatment (TARGET QUADRANT: $F_{3,36}$ = 0.37, n.s).

Distance Travelled: The distance travelled in the four quadrants was different (QUADRANTS: $F_{3,105}$ = 3.95, p<0.01; d*ata not shown*) and this difference was not affected by metrifonate treatment (QUADRANTS by TREATMENT: $F_{9,105}$ =1.35, n.s.). The bias for the quadrant in which the platform had been positioned during training was not influenced by metrifonate treatment (TARGET QUADRANT: $F_{3,36}$ = 86, n.s).

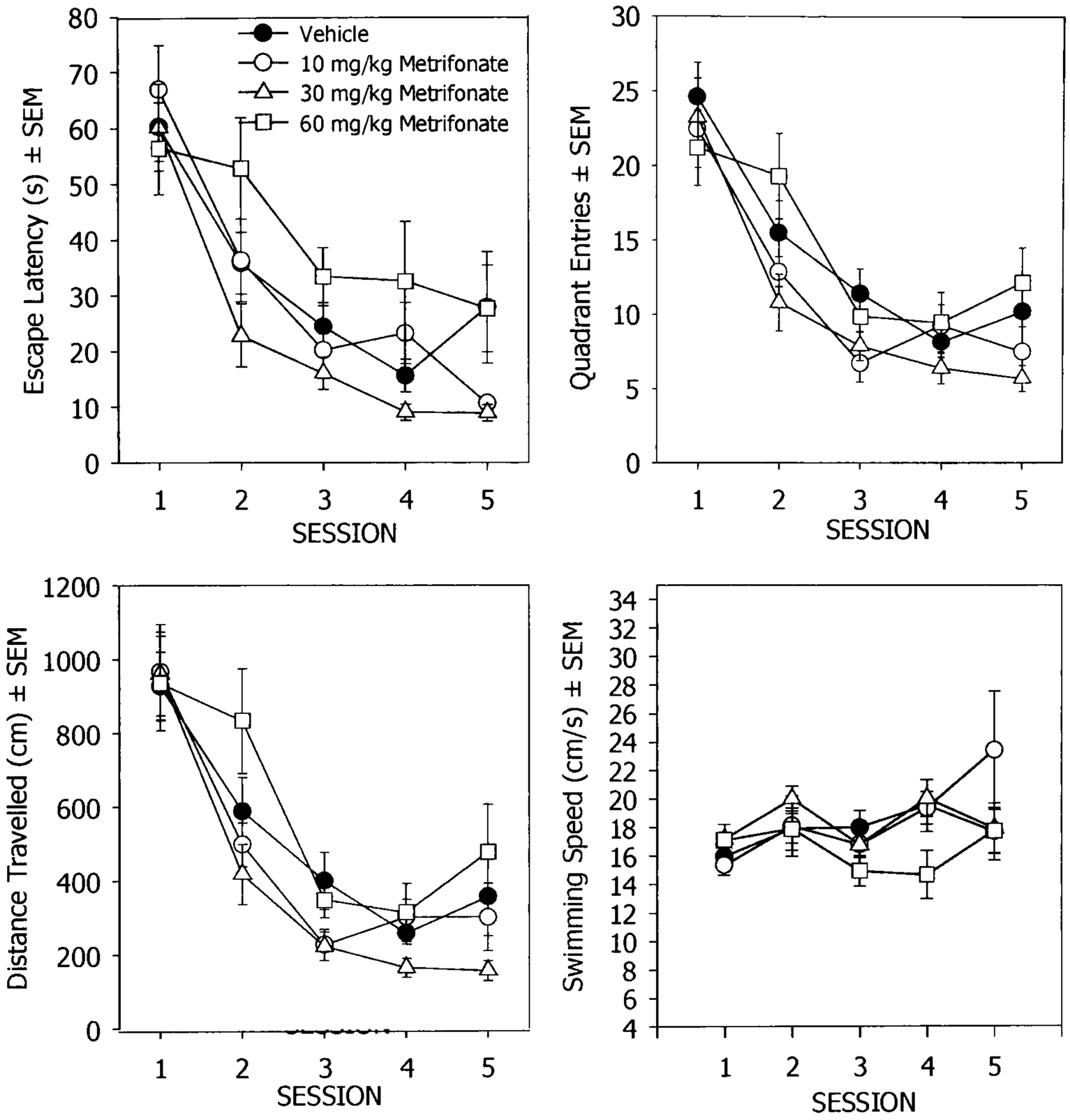


Figure 7: Effect of Sub-chronic metrifonate (10, 30 and 60 mg/kg) on the performance in the water escape task in young male C57BL Mice (N= 10 per group). Session means and standard errors of the means (SEM) are depicted for latencies (s) to escape onto a platform (*upper left panel*), number of quadrant entries (*upper right panel*), distance travelled (cm; *lower left panel*) and swimming speed ($cm*s^{-1}$; *lower right panel*)

Time in Annulus: Metrifonate treatment did not affect the time spent in the annulus region of the maze ($F_{3, 36}$ = 0.68, n.s; see figure *8, Left panel*)

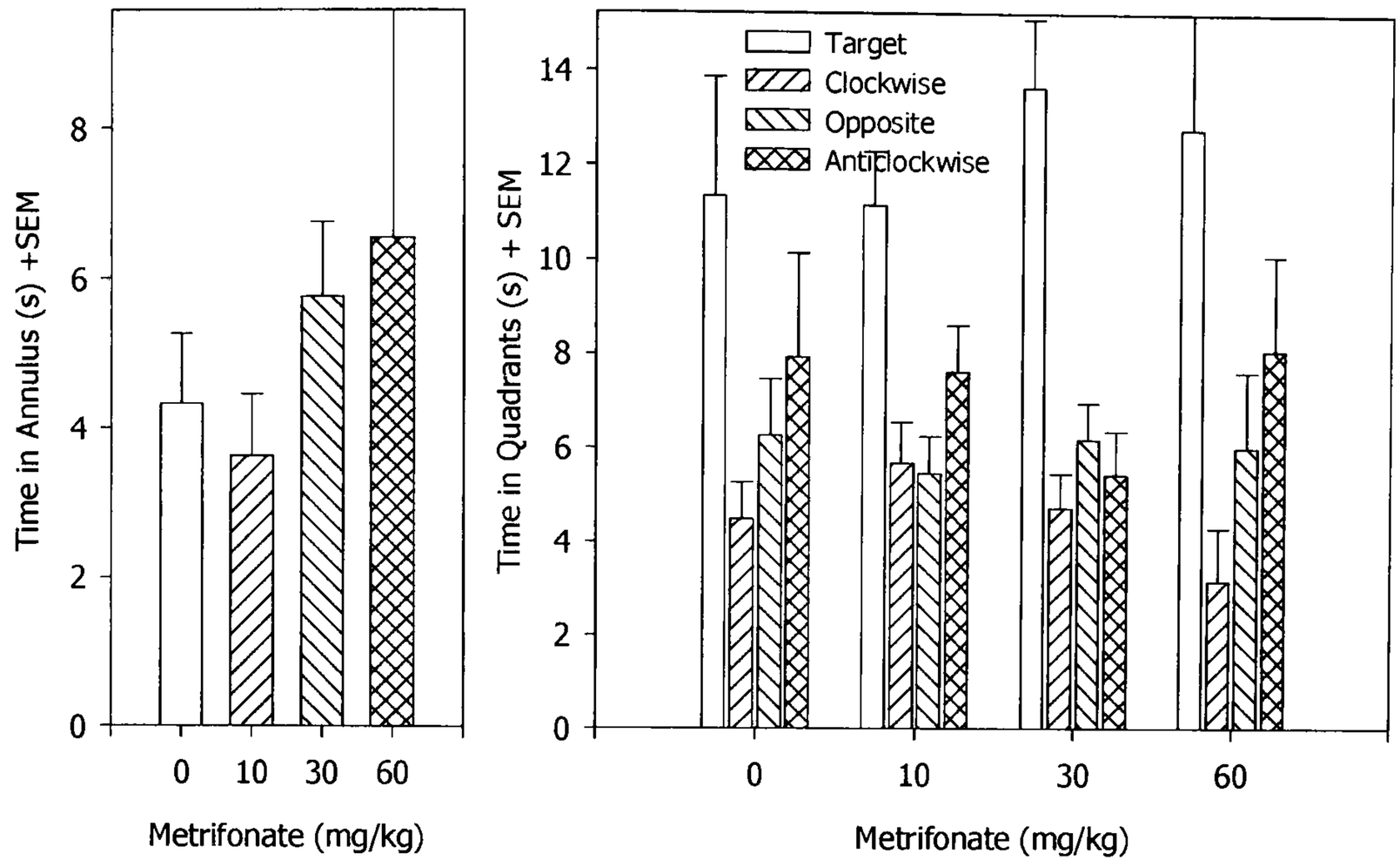


Figure 8: Effects of sub-chronic metrifonate (10, 30 and 60 mg/kg) on the performance in the water escape task probe trial young male C57BL Mice (N= 10 per group). Session means and standard errors of the means (SEM) are depicted for time spent in the Annulus (s) (*left panel*) and time spent in the quadrant (*right panel*)

Discussion

Effects of metrifonate inhibitors in the rat

In this study metrifonate, at all doses tested, failed to induce significant improvement in spatial reference memory in neurologically normal rats (section 5.1)

The lack of improvement in learning in the Morris water maze escape task by metrifonate could be explained by the fact that intact young rats do not suffer from memory impairments or cholinergic hypofunction. Multiple doses of metrifonate increase and prolong the effect on ACh levels in the brain (Hallak and Giacobini, 1989) and this increased ACh concentration in the synaptic cleft will induce agonistic effects postsynaptically. However, presynaptic action may also occur, blocking the mechanism for choline uptake and thus slowing down the rate of synthesis and release of ACh. In the intact rat with no existing cholinergic deficit chronic doses may mask the beneficial effects of cholinesterase inhibition by presynaptical blockade (Nordgren, 1992).

However previous studies have shown that cognitive improvement in various animal models (Blokland et al, 1995; van der Staay et al, 1996a,b; Kronforst et al 1995) was not diminished after subchronic administration. One possible explanation for the lack of effect of metrifonate on behaviour in the Morris water escape task is the high performance level of the young rats in this study may have imposed a ceiling effect (i.e. the rats performed at a that masked) any performance improving effect of metrifonate.

Possible biological variability or differences in sensitivity of the rats to the treatment with the compound may also have obscured the efficacy of the continuous metrifonate regimen compared to previously reported results (van der Staay et al, 1996a,b). Although the two groups of rats were tested under highly standardised conditions, there were extremely different performances in the probe trials. The Wistar strain used was an outbred strain in which the genome is not fixed, as is in the case of inbred strains and their F_1 crosses. The changes in performance between the two experiments could be due to genetic drift between the two different shipments of animals (van der Staay, 2000).

Metrifonate in the mouse

The behaviour of mice in tasks of learning and memory is less documented than in rats. In view of the development of transgenic mouse models with predefined deficiencies as new tools to identify putative cognition enhancers, it is important to conduct preliminary tests on parental strains that are used to produce these transgenic animals, in order to calibrate the behavioural paradigm before beginning testing of the transgenic or knockout mice. As the acute and of sub- chronic experiment using rats as subjects, this study looked at the effects of acute and sub-chronic metrifonate on performance in the Morris water escape task using the wild type C57BL 6J mouse strain. This mouse strain has previously been shown to readily learn the platform escape response in the Morris water tank (Paylor et al, 1990; Finkelstein et al 1994)

The cognitive testing performed in this present study confirmed that the wild type of C57BL mice performs well in the Morris water maze. The administration of metrifonate, however, induced no improvements in spatial reference memory in these neurologically normal mice. This confirms previously reported results (Ikonen et al, 1999) of a lack of effect of metrifonate treatment in intact mice.

With a pre-treatment regimen of three weeks before the testing began metrifonate showed beneficial effects at the dose of 30 mg/kg with the mice escaped to the platform faster than the control group. However an impairment was observed in learning performance with the higher dose of metrifonate (60 mg/kg) this was most probably due to first cholinergic side effects, which, however, were not overt during the behavioural testing.

As discussed in chapter 4 repeated administration of metrifonate to rats or rabbits accumulates this long lasting cholinesterase inhibitor resulting in a stable level, thereby decreasing the fluctuations between peak and trough ChE inhibition seen after each single dose. Therefore it is a possibility that the long lasting inhibition of cholinesterase induced by metrifonate may contribute to the improvement in the mouse Morris water escape task compared to that of the acute study in the mouse.

Normal Animal Model

The use of normal animals makes the assumption in that the subjects perform suboptimally and that there is room for improvement. Moreover, it makes the implicit assumption that if a compound that is active in normal subjects then it would also be useful for the treatment of patients (Gamzu, 1985). A problem of assessing cognitive enhancing potential in normal animals is that improved performance in normal animals may not necessarily predict efficacy of the drug in AD patients. Moreover, no effects are observed in normal animals, the compound might still ameliorate cognitive impairments in patients (Decker, 1995).

The most important point is that compounds that improve cognitive function in normal animals may be classed as cognitive enhancers. However, because there is no clinical symptom of AD in these animals, the potential of a compound as disease modifiers remains unknown.

Further Study

Based on literature results, there is a strong correlation between the cholinergic system and cognitive performance. Cognitive impairment as seen in AD is related to cholinergic hypofunction (Bartus et al, 1982; Bierer et al, 1995). To further assess the effects of cholinesterase inhibitors designed for the treatment of AD it is necessary to use animals with naturally occuring or experimentally induced cholinergic hypofunction. For example, the use of pharmacological agents such as scopolamine or lesions of cholinergic structures that could induce cognitive impairments in the rodent is indicated. The effects of the cholinesterase inhibitors could then be further characterised in both the rat and the mouse.

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Acute Metrifonate in the Morris Maze with Scopolamine Induced Amnesia.

Abstract

We investigated the effects of acute pre-training treatment of the cholinesterase inhibitors metrifonate on water maze spatial navigation in mice with scopolamine-induced amnesia.

Scopolamine (1 mg/kg) impaired acquisition of the Morris water escape task: it induced an increase in escape latency. Retention was also impaired: as the scopolamine treated mice spent less time in the annulus region of the maze. Metrifonate (10 and 30 mg/kg) had no ameliorating effects on the scopolamine-induced amnesia.

Introduction

The Morris Maze task (Morris, 1984) is one of the most frequently used experimental paradigms designed to assess cognitive performance. Most studies have used rats as subjects, whereas mice have been less frequently used (Paylor, et al 1993; Sweeney, et al, 1988 and van der Staay, 2000). Mice have been shown to produce a normal learning curve (van der Staay et al, 2000; Klapdor, & van der Staay, 1996).

The behaviour of mice in tasks of learning and memory is less characterised than in rats and in view of the development of transgenic mouse models with predefined deficiencies as new tools to identify putative cognition enhancers, it is important to conduct preliminary tests on wild type strains that are used to produce these transgenic animals, to calibrate the behavioural paradigm before beginning testing of the transgenic or or knockout mice.

Scopolamine, a muscarinic antagonist that has been shown to impair behaviour in a variety of tasks (Buresova *et al*, 1986; Rush, 1988) and has been used as a model of ageing and dementia producing some of the cognitive impairments seen in AD

(Wesnes et al, 1991). Once having confirmed that scopolamine treatment causes deficits in the Morris maze task, future study would involve testing cognitive enhancers in this model to try to ameliorate this scopolamine induced deficit.

The aim of this study is determine the effects of metrifonate on scopolamine-induced amnesia in the mouse

Procedure

Animals: male C57Bl/6J mice, aged about 10 weeks, weighing approximately 20-25 grams, were supplied by Winkelmann (Borchen, Germany). The animals were housed in groups of eight in standard Makrolon™ type III cages. They were kept under and artificial 12-hour light/12 hour dark regimen (lights on from 7.00 to 19.00) in a temperature (ca. 21.5°C) and humidity (50%) controlled animal room. Water and food were available ad libitum. Before testing the animals were transferred to the experimental room where they were housed for the duration of the testing period. Housing conditions were similar to those in the animal room.

Apparatus and Methods: The water tank used was a circular grey tank (polyethylene) with a slightly sloping wall (inner dimensions: diameter at top 74cm, diameter at bottom 66 cm, depth 54 cm.) The tank was filled with clear tap water at a temperature of approximately 22°C. The escape platform was a grey polyethylene cylinder (7.3 cm width) submerged 0.6cm below the surface of the water.

The tank was situated in a room illuminated by white fluorescent tubes. The lights directly above the maze were turned off to prevent reflection. Blinds were closed to prevent the entrance of natural light. Extra maze cues were provided by the furniture in the room, including desks, computer equipment, the presence of the experimenter and by a radio on a shelf that was playing softly. All testing was done between 9.00 and 14.00

The video camera, mounted in the centre above the circular pool, provided a picture of the pool on a TV monitor. The movements of the mice were registered using the automatic video tracking system EthoVision™.

The animals received four trials daily, on five successive days (Morris, 1984). A trial was started by placing a mouse into the pool, facing the wall of the tank. Each of the four starting positions (arbitrarily assigned north, south, east, west) was used in a series of four trials; their order was randomised. The escape platform was always in the same fixed position in the west quadrant. A trial was terminated as soon as the mouse had climbed onto the escape platform or when 90 seconds had elapsed, whichever event occurred first. Each mouse was allowed to stay on the platform for 30 seconds. Then it was taken from the platform and the next trial started immediately. Mice that did not find the platform within the 90 seconds were put on the platform by the experimenter and were allowed to stay there for 30 seconds.

After the fourth trial on the fifth session, an additional trial was given as a probe trial: the platform was removed, and the time spent in the four quadrants was measured for 30 seconds. The area around the platform was defined as the annulus region. On the probe trial, all mice started from the same position, opposite to the quadrant where the escape platform had been positioned during acquisition.

Drug Administration: Metrifonate was solved in Sodium citrate buffer, pH 5.5 and applicated daily per os (p.o) 30 minutes prior to the start of the trials in an application volume of 20ml/kg. Scopolamine was solved in 0.9% NaCl solution and was applicated interperitoneally (i.p.) in an application volume of 10 ml/kg. Scopolamine was administered in conjunction Metrifonate, 30 mins prior to training. For the doses and number of animals per group/ per compound see table 1.

Table 1: Number of animals per group and treatment combinations (doses in mg/kg) in three experiments designed to study the effects of the ChEI's metrifonate, rivastigmine and donepezil on scopolamine-induced deficits in mice.

	Group			
	1	2	3	4
A	N = 10	N = 10	N = 8	N = 10
Metrifonate	0 (Vehicle)	0 (Vehicle)	10	30
Scopolamine	0 (Vehicle)	1	1	1

Analysis: EthoVision™ analysed the distance travelled, the distance to point (platform), the time spent in zone and the velocity of the animal. The mean per mouse, per trial was then calculated. This data was then analysed per mouse per session by the SAS GLM-procedure. The following four parameters for acquisition of the water escape task were analysed

Statistically: the escape latency (in s); number of quadrant entries; the distance travelled (in cm); and the swimming speed (in cm/s). They were assessed with a TREATMENT*SESSIONS analysis of variance (ANOVA) with repeated measures over SESSIONS. Analysis was supplemented by T test post hoc comparisons. A difference between groups was considered significant if the p value was below 0.05. Group differences in the time spent in the annulus during the probe trial was assessed by ANOVA. Drug effects on the time and distance travelled in the quadrant was assessed with a repeated measures ANOVA over QUADRANTS. Group differences were further evaluated by T test post hoc comparisons ($p< 0.05$). The scopolamine verses vehicle group were initially analysed separately to determine the effects of scopolamine alone.

Results

Scopolamine

Escape latency: All mice started from the same level of performance (First SESSION: $F_{1,14} = 1.83$, n.s). Averaged over all sessions, scopolamine had an influence on the escape latency (GENERAL MEAN: $F_{1, 14} = 87.56$ $p<0.01$ see figure 1, *Upper left panel*). The escape latencies of the mice decreased in the course of training (SESSIONS: $F_{4,72}= 36.07$, $p<0.01$), and this rate of learning was affected by scopolamine treatment ($F_{4,72} = 2.81$, $p<0.05$).

Quadrant Entries: The mice all started from the same level of quadrant entries (First Session: $F_{1,14} = 0.45$, n.s). Scopolamine influenced the mean number of quadrants entered during swimming, averaged over al sessions (GENERAL MEAN: $F_{1,14} = 44.28$, $p<0.01$, see figure 1, *Upper right panel*). During training the mice reduced their quadrant entries (SESSIONS:$F_{4,72} = 12.97$, $p<0.01$) and though the rate of improvement during the sessions appeared to be similar for both groups (SESSIONS by TREATMENT: $F_{4,72} = 1.75$, n.s).

Distance Travelled: The path lengths to escape onto the platform were all similar at the start of training ($F_{1,14}$= 0.64, n.s). Averaged over all sessions, scopolamine treated swam a longer distance (GENERAL MEAN: $F_{1,14}$ = 48.67, $p<0.01$; see figure 1, *Lower left panel*). All mice reduced the distance swum over the sessions (SESSIONS:$F_{4,72}$ = 6.00, $p<0.01$). The reduction in the distance travelled during the sessions appeared to be similar for both groups (SESSIONS by TREATMENT: $F_{4,72}$ = 1.72, n.s).

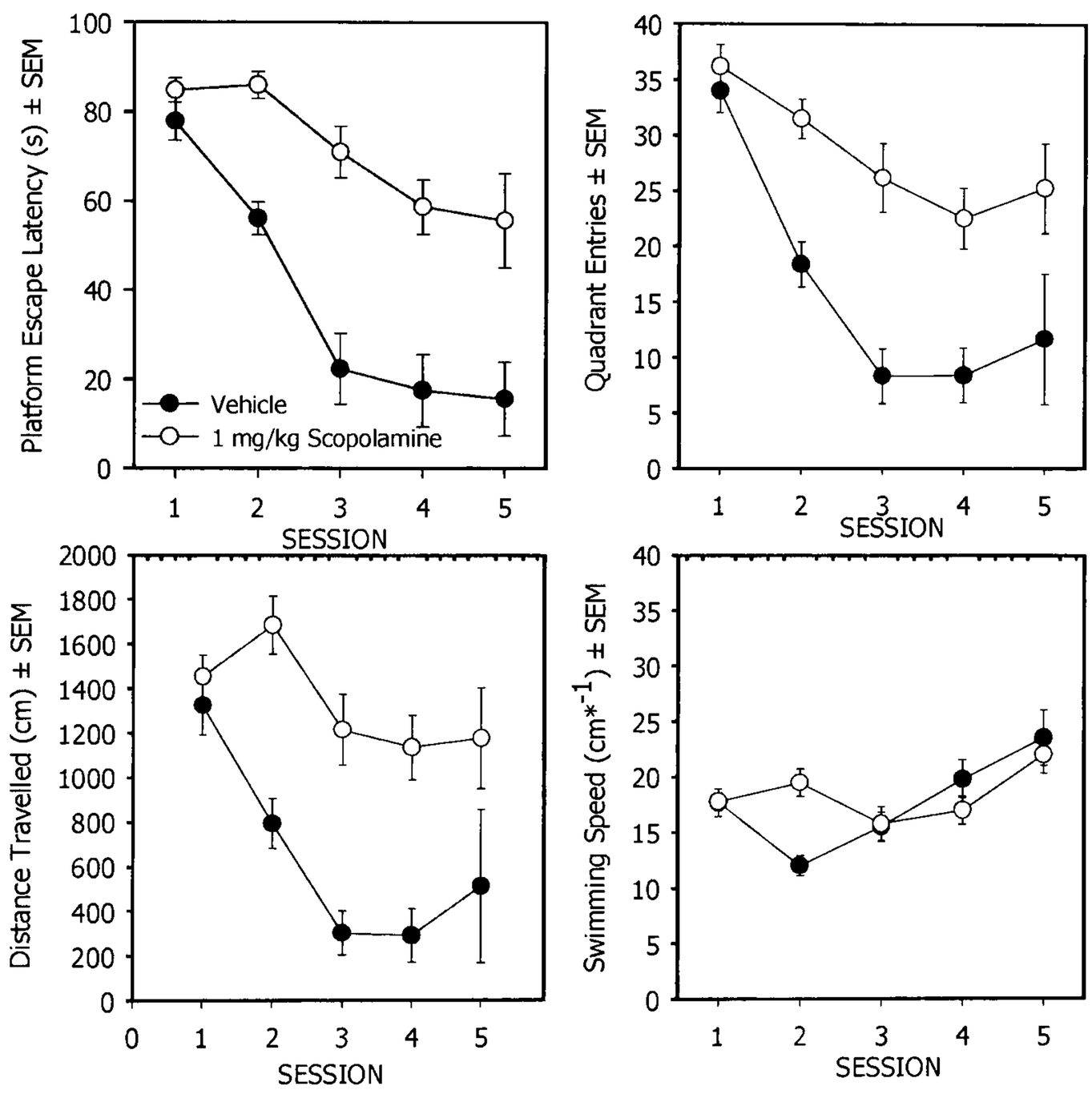

Figure 1: Effect of acute scopolamine (1 mg/kg) on the performance in the water escape task by in treated young male C57/BL Mice (N= 8). Session means and standard errors of the means (SEM) are depicted for latencies (s) to escape onto a platform (*upper left panel*), number of quadrant entries (*upper right panel*), distance travelled (cm; *lower left panel*) and swimming speed (cm^{*-1}; *lower right panel*)

Swimming Speed: Averaged over all sessions, scopolamine had no influence on the swimming speed (GENERAL MEAN: $F_{1,14}$ = 0.32, n.s., see figure 1, *Lower right panel*). The swimming speed of the mice changed in the course of training (SESSIONS: $F_{4,72}$= 8.52, $p<0.01$), this was affected by scopolamine treatment (SESSIONS by TREATMENT: $F_{12,104}$ = 3.93, $p<0.05$). Further analysis per session

revealed that during session 2 the swimming speed was increased by scopolamine compared to the speed of the vehicle treated mice.

Probe Trial.

Time in Quadrant: There was a slight difference in the time the groups spent in the four quadrants (QUADRANTS: $F_{3, 54} = 2.37$, $p<0.06$; *data not shown*). The bias for the quadrant in which the platform had been positioned during training was influenced by scopolamine treatment (TARGET QUADRANT: $F_{1, 18}= 4.22$, $p<0.05$); i.e. the scopolamine treated mice spent less time in the target quadrant than vehicle treated mice.

Distance Travelled: The distance travelled in the four quadrants was different (QUADRANTS: $F_{3,54} = 4.41$, $p<0.05$) and tended to be affected by the scopolamine treatment (QUADRANTS by TREATMENT: $F_{3, 54} = 2.40$, $p<0.07$). The bias for the quadrant in which the platform had been positioned during training was not influenced by scopolamine treatment (TARGET QUADRANT: $F_{1,18}= 2.63$, n.s). The scopolamine treated mice tended to travel a longer distance in the target quadrant than vehicle treated mice.

Time in Annulus: Scopolamine treatment did affect the time spent in the annulus region of the maze ($F_{1,18}= 9.09$, $p<0.01$; see figure 2), Scopolamine treated mice spending less time in the annulus than the vehicle controls.

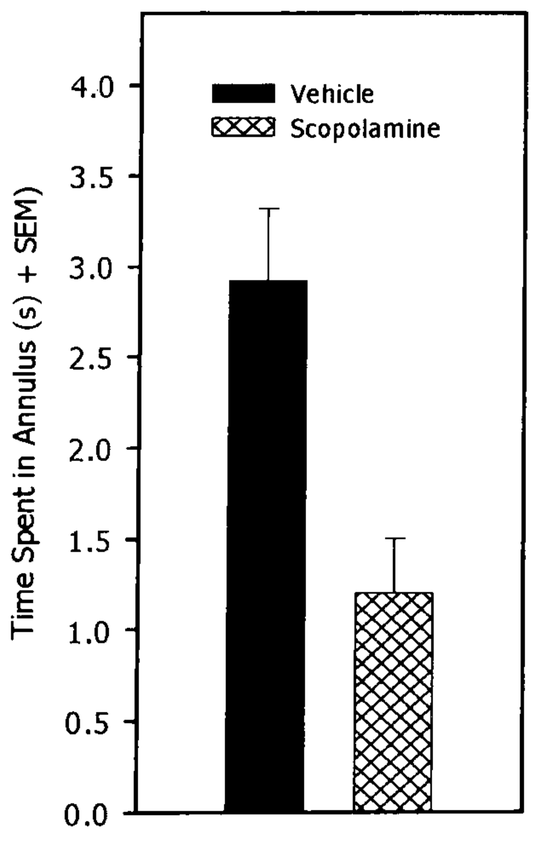

Figure 2: Effects of acute Scopolamine (1 mg/kg) on the performance in the water escape task probe trial with young male C57/BL Mice (N= 8) on the time spent in the Annulus (s)

Results

Effects of metrifonate in scopolamine induced amnesia in the mouse

Acquisition Trials

Escape latency. Mice started from a slightly different level of performance ($F_{3,34}$ = 2.95, $p<0.05$, See figure 3 upper left panel). Averaged over all sessions, treatment with metrifonate and or scopolamine had an influence on the escape latency (GENERAL MEAN: $F_{3,34} = 60.52$, $p<0.01$). Post hoc analysis revealed that the

scopolamine treated group and the metrifonate/scopolamine treated groups had a longer escape latency compared to that of the vehicle treated group. The metrifonate treated groups also had a longer escape latency compared to the scopolamine treated group. The escape latencies of the mice decreased in the course of training (SESSIONS: $F_{4,136} = 18.01$, $p<0.01$), and this rate of learning was affected by metrifonate and or scopolamine treatment (SESSIONS by TREATMENT: $F_{12,136} = 3.56$, $p<0.01$).

Quadrant Entries: The mice all started from the same level of quadrant entries ($F_{3,34}$ = 2.42, n.s). Over all sessions the treatment of metrifonate and/ or scopolamine influenced the mean number of quadrants entered during swimming (GENERAL MEAN: $F_{3,34} = 31.58$, $p< 0.001$; see figure 3, *Upper right panel*). During training the mice reduced their quadrant entries (SESSIONS: $F_{4,136}= 17.91$, $p<0.01$). The rate of improvement during the sessions was similar for all groups (SESSIONS by TREATMENT: $F_{12,136} = 1.35$, n.s.).

Distance Travelled: The path lengths to escape onto the platform were all different at the start of training ($F_{3,36} = 8.32$, $p<0.01$). Averaged over all sessions, animals exposed to metrifonate and or scopolamine had a longer mean distance travelled during training (GENERAL MEAN: $F_{3,34} = 32.08$, $p<0.01$., *see figure 3, Lower left panel*). All mice reduced the distance swum over the sessions (SESSIONS:$F_{4,136}$= 8.52, $p<0.01$) however, the rate of reduction over the sessions was similar for all groups (SESSIONS by TREATMENT: $F_{12,136} = 1.27$, n.s).

Swimming Speed: The swimming speed of the mice differed at the start of training ($F_{3,34}$ = 5.49, p<0.01). Averaged over all sessions, Metrifonate and or scopolamine treatment had an influence on the swimming speed (GENERAL MEAN: $F_{3,34}$ = 3.72, p<0.05., *see figure 3, Lower right panel*). The swimming speed of the mice changed in the course of training (SESSIONS:$F_{4,136}$= 9.34, p<0.01), this was not affected by metrifonate or scopolamine treatment (SESSIONS by TREATMENT: $F_{12,136}$= 1.74, n.s.). However further post hoc analysis revealed that 30 mg/kg metrifonate treated group slightly increased the swimming speed compared to vehicle and scopolamine treated groups over sessions.

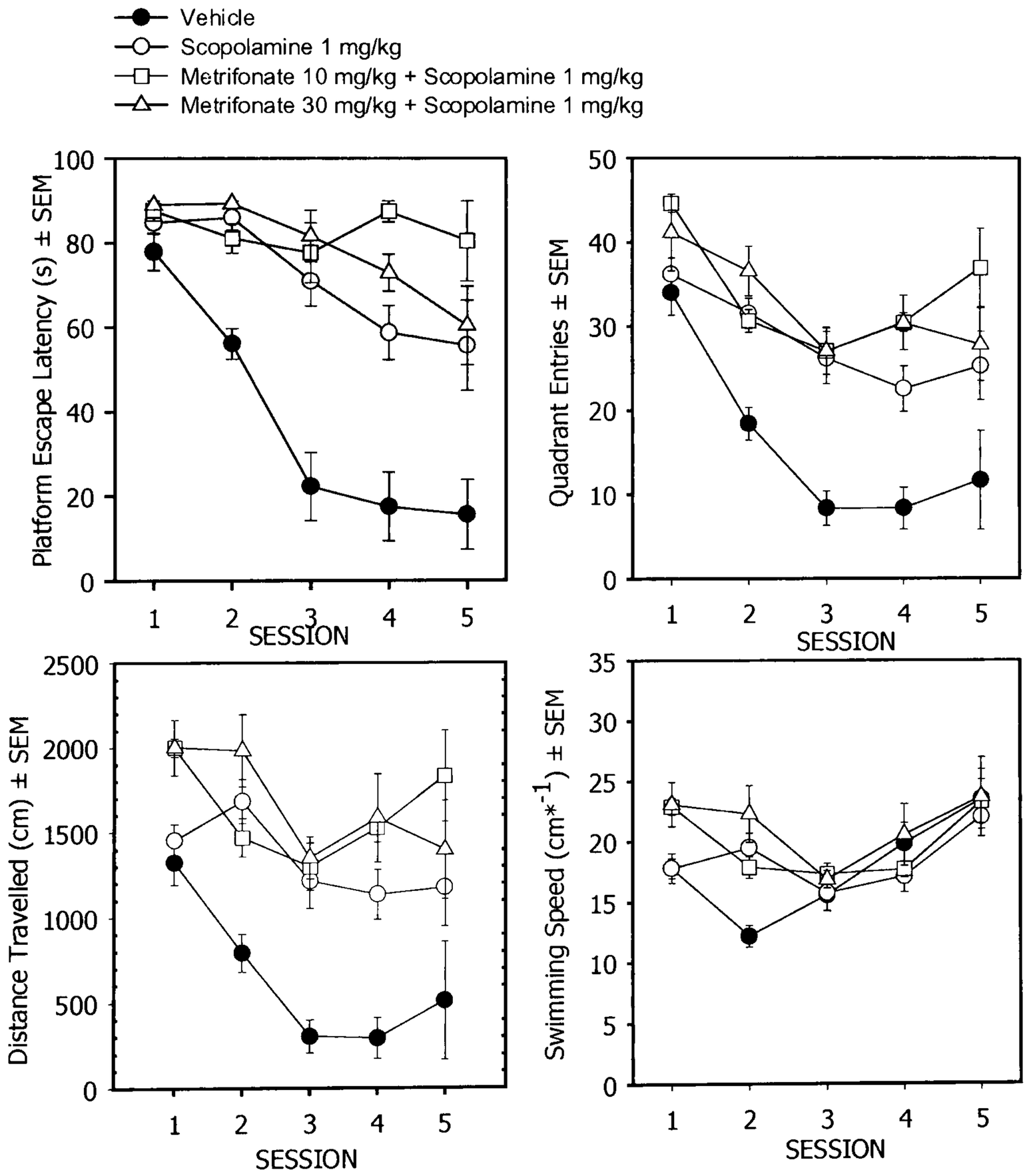

Figure 3: Effect of acute metrifonate (10 [N = 8] and 30 mg/kg [N = 10]) on the performance in the water escape task in scopolamine (1 mg/kg [N= 10]) treated young male C57/BL Mice. Session means and standard errors of the means (SEM) are depicted for latencies (s) to escape onto a platform (*upper left panel*), number of quadrant entries (*upper right panel*), distance travelled (cm; *lower left panel*) and swimming speed ($cm*s^{-1}$; *lower right panel*)

Probe Trial

Time in Quadrant: The time spent in the four quadrants was not different (QUADRANTS: $F_{3, 102} = 1.12$, n.s.; *see figure 4, Right panel*), However, the bias for the quadrant in which the platform had been positioned during training was influenced by drug treatment (TARGET QUADRANT: $F_{3, 34} = 3.88$, $p<0.05$). Post hoc analysis revealed that the 10 and 30 mg/kg treated groups spent less time in the training quadrant than the vehicle treated group.

Distance Travelled: The distance travelled in the four quadrants was different (QUADRANTS: $F_{3,102} = 7.69$, $p<0.001$; *data not shown*) and this difference tended to be affected by drug treatment (QUADRANTS by TREATMENT: $F_{9,102} = 1.92$, $p<0.07$). The bias for the quadrant in which the platform had been positioned during training tended to be influenced by drug treatment (TARGET QUADRANT: $F_{3,34} = 2.72$, $p<0.06$). Post hoc analysis revealed that the 10 and 30 mg/kg treated groups swam a shorter distance in the target quadrant than did the vehicle treated group.

Time in Annulus: The time spent in the annulus region of the maze was affected by drug treatment ($F_{3,34} = 12.73$, $p<0.001$; *see figure 4, left panel).* Post hoc analysis revealed that the 10 and 30 mg/kg metrifonate treated groups and the scopolamine treated group spent less time in the annulus region than the vehicle treated group.

NB/ It is noted that there is only slight or no differences of the time spent in the quadrants or the distance travelled in the vehicle treated group, indicating that the mice did not fully learn the task. However, taken together with the time spent in the annulus region of the maze, and the bias for the target quadrant do suggest that the vehicle treated mice did achieve some level of learning to find the platform position.

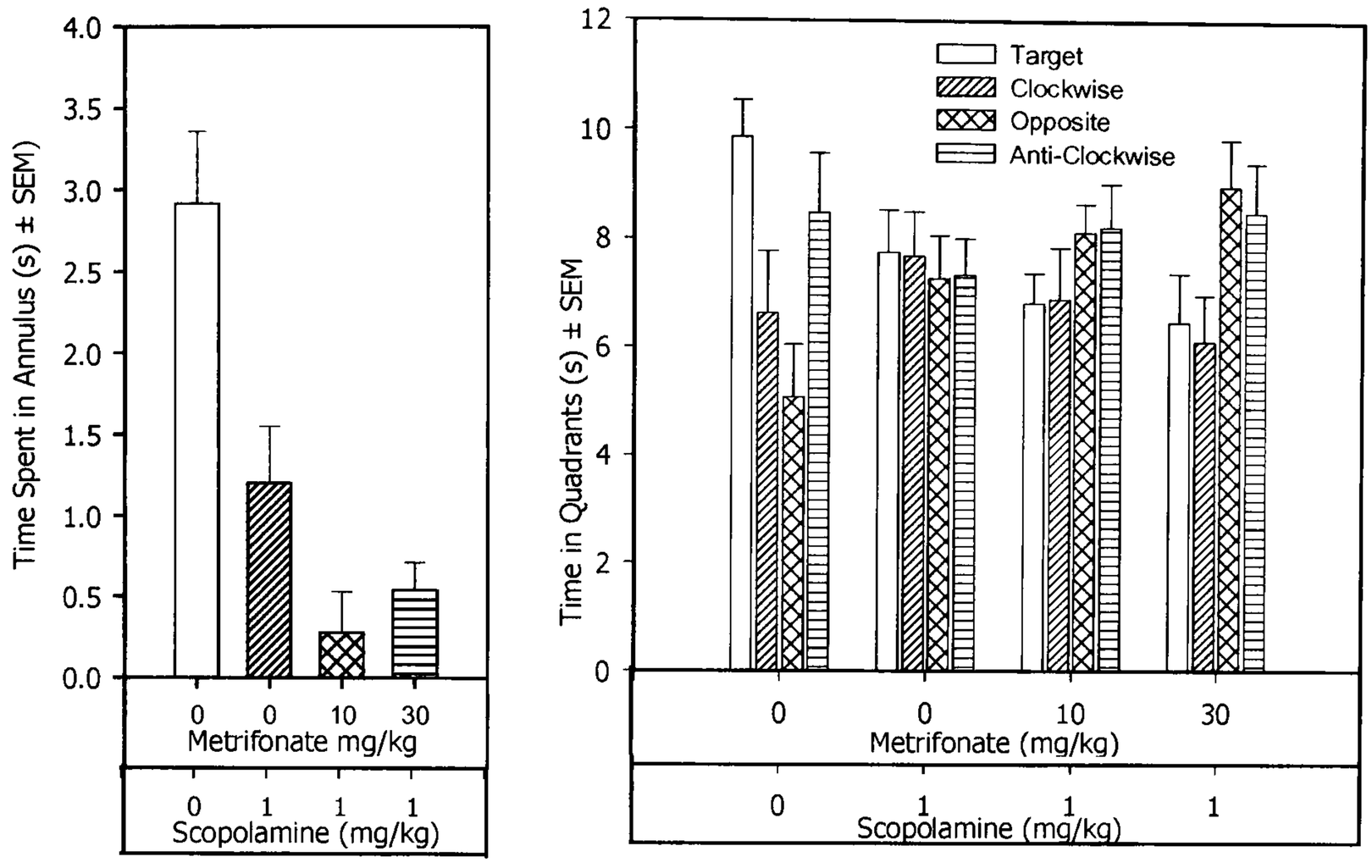

Figure 4: Effects of acute metrifonate (10 and 30 mg/kg) on the performance in the water escape task probe trial by scopolamine (1 mg/kg) treated young male C57/BL Mice. Session means and standard errors of the means (SEM) are depicted for Time spent in the Annulus (s) (*left panel*) and time spent in the quadrant (*right panel*)

Discussion

Summary of results

Scopolamine interferes with memory and cognitive function in humans (Beatty et al, 1986) and animals (Stevens, 1981; Sutherland et al., 1982) by blocking muscarinic receptors. The present study compared the effects of the cholinesterase inhibitor metrifonate, on scopolamine-induced memory impairments in the Morris water maze.

Scopolamine (1 mg/kg) impaired performance in the Morris water escape task by having an increase in escape latency during acquisition of the task. Retention was also impaired as seen by less time spent in the annulus region of the maze. Metrifonate (10 and 30 mg/kg) had no ameliorating effects on the scopolamine-induced amnesia. The inability of metrifonate to alleviate memory deficits induced by scopolamine may be related to excessive stimulation of the cholinergic system.

Swimming behaviour

The Morris water escape task has been designed to measure spatial learning and memory (Morris, 1984). Exploration is the first step in the spatial navigation of the

environment and in normal healthy rodents the occurrence of exploration decreases with the time spent in novel surroundings.

The vehicle treated mice swam away from the wall, searched the inner areas of the pool, and found the hidden platform on nearly every trial. They also often swam directly from the start point to the hidden platform particularly during later sessions.

The scopolamine treated mice on the other hand, displayed thigmotaxic swimming. They swam predominantly near the maze wall and failed to find the hidden platform during the trials (See figure 5). This thigmotaxic swimming pattern is a common behaviour in rats given NMDA or muscarinic antagonists (Paylor et al, 1990; Whishaw et al, 1987). The scopolamine + metrifonate treated rats showed similar thigmotaxic swimming. However, no metrifonate effect could be observed due to the excess cholinergic stimulation with the combination drug administration. In chapter 5, metrifonate alone caused no obvious changes in swimming behaviour compared to vehicle treated animals (see figure 5, *lower panel*).

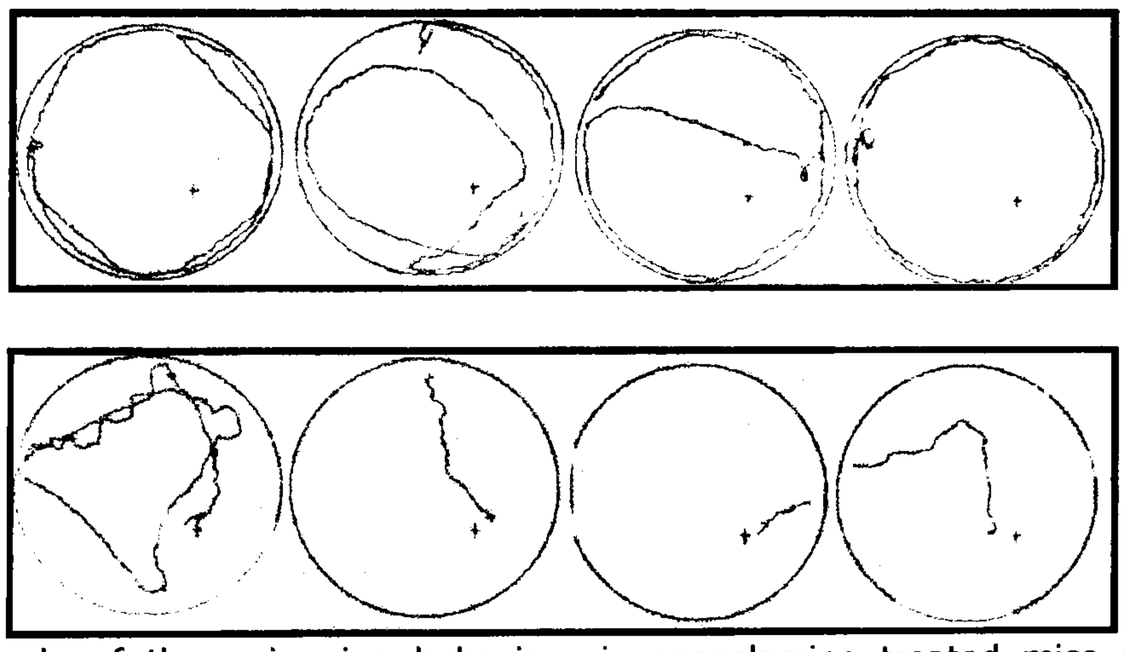

Figure 5: An example of the swimming behaviour in scopolamine treated mice (*upper panel*) and vehicle treated mice (*lower panel*) during four successive days of training in the standard Morris water escape task

Other disturbances have previously been observed by Cain and Saucier (1996) in rats and in this study in mice, including deflecting off the hidden platform when it was contacted, followed by swimming away (deflections) and failure to climb onto the platform when they swam close to it (Passes). The animals also failed to remain on the platform when placed there. Instead they jump off and continue to swim thigmotaxically.

These sensory motor disturbances are thought to be the reason for poor acquisition scores because if an animal spent most of its time swimming thigmotaxically, and

deflected of the platform and failed to remain on it when placed there, it would be unlikely to learn much about its spatial location. Visual ability is an important factor in the water maze task and NMDA and muscarinic antagonists have been reported to disrupt visual discrimination performance in other tasks (e.g. non spatial operant discrimination task; Clissold et al, 1991; cue discrimination; Rick et al, 1981). However, Morris (1986 and 1989) and Cain (1996) showed that NMDA and muscarinic antagonists did not interfere with simple discriminations or the learning phase of a visible discrimination task but did interfere with the performance.

Therefore it can be said that muscarinic antagonists reduce the amount of information about platform location that animals acquire while in the maze. However, this does not indicate that sensorimotor disturbances cause low maze acquisition, rather that the antagonists cause sensorimotor disturbances and maze deficits by unrelated but parallel effects on sensorimotor and learning mechanisms.

Pre-training the animals is one way to separate sensorimotor deficits and learning to search for, use and remain on the hidden platform (Morris, 1989; Whishaw et al, 1987). In pre-trained animals, sensorimotor deficits were absent allowing the animals to behave effectively in the maze task. However, pre-training does not eliminate increased platform search time in drugged animals (Whishaw, 1989). Therefore, spatial deficits induced by a muscarinic antagonist are still present and still an effective method if one tries to demonstrate that cholinesterase inhibitors are able to ameliorate experimentally induced cognitive deficits. Finally, the vehicle treated mice did not perform well in the probe trial, showing only slightly better performance to that of the scopolamine treated animals. Future studies with mice need to ensure good behaviour from vehicle treated/normal use animals so that any drug effects can be studied more effectively.

Conclusions and further study

The use of a cholinesterase inhibitor scopolamine-induced impairment is merely a measure of in vivo muscarinic agonism and although scopolamine models are capable of indicating something about the interaction of a compound with the cholinergic system, the nature of the interaction and the ability of the compound to overcome the type of cholinergic hypofunction in AD are unclear. Another problem with cholinergic antagonist models that produce deficits through pharmacological manipulations is the possibility of pharmacokinetic interactions, and though in this

study with scopolamine and the cholinesterase inhibitors used it is a remote possibility that such interactions occur, other alternatives need to be studied.

One such alternative is excitotoxic lesions, which can be aimed at more specific areas of the brain involved in learning and memory rather than the general effect of scopolamine, which inhibits muscarinic receptors throughout the entire brain.

Spatial Discrimination Deficits by Excitotoxic Lesions in the Morris Water Escape Task

Abstract

The effects of the cholinesterase inhibitor (ChEI) metrifonate was assessed on spatial performance of rats with bilateral lesions of the entorhinal cortex (EC). EC-lesioned rats model early changes in the brains of patients suffering from Alzheimer's disease. In the present study, we found that spatial discrimination deficits in rats, induced by bilateral ibotenic acid (IBO) lesions of the EC region can partially be antagonised by treatment with metrifonate (30 mg/kg). Performance was improved in the spatial discrimination task compared with that of the EC lesioned control group. It is concluded that the rat with bilateral EC lesions is a suitable deficit model for the assessment of effects of putative Alzheimer therapeutics.

Introduction

The hippocampus proper, the entorhinal cortex and the subiculum are intimately connected and are described as the major components of the hippocampal formation (Amaral *et al*, 1989). The hippocampal formation is that it is involved in learning and memory processes (O'Keefe *et al*, 1978; Rudy *et al*, 1989). The entorhinal cortex receives information from the neocortex and sends major projections to the hippocampus by way of the trisynaptical or perforant pathway (Witter *et al*, 1989). Cognitive dysfunctions observed after EC damage are mainly considered to be due to hippocampal deafferentiation. Post-mortem studies in the brains of Alzheimer's patients at a very early stage of the disease reveal that damage to the EC occurs and this early EC damage correlates with the memory deficits present during the early stage of Alzheimer's disease (Braak and Braak, 1991; Hyman *et al*, 1986; Miwa & Ueki, 1996).

An initial theory by O'Keefe and Naldel (1978) is that the hippocampal formation is involved in spatial memory. In rats, bilateral damage to the EC resulted in spatial learning impairments, similar to impairments due to disturbed hippocampal functioning (Rasmussen *et al*, 1989; Fugger *et al*, 1997; Eijkenboom *et al*; 2000). Electrophysiological data has demonstrated a direct influence of the EC in spatial learning and memory, as some cells within the EC fire when monkeys (Rolls *et al*, 1989) or rats (Barnes *et al*, 1990) are positioned in a specific area.

Spatial learning deficits induced by entorhinal cortex lesions could be reversed by a positive modulator of the AMPA receptor, aniracetam (Zajaczkowski and Danysz, 1997), by NMDA receptor antagonists, (+)-MK-801 and memantine (Zajaczkowski *et al*, 1996), and by ganglioside (Ramirez *et al*, 1991; Ramirez *et al*, 1998), suggesting a sensitivity of this deficit model for pharmacological interventions with putative and established Alzheimer therapeutics.

A reduction in acetylcholinesterase positive fibres in the CA3 and stratum moleculare of the hippocampal formation has been found in the rat hippocampus after entorhinal cortex lesions (Miwa and Ueki, 1996). In the dentate gyrus, there was a 45 percent decrease in acetylcholine after the EC lesion and a 41 percent decrease was found in the CA3. These results suggest that the EC lesion induced cognitive deficits are due to indirect damage to the hippocampal formation (Hunt *et al*, 1994; Skelton *et al*, 1992; Jarrard *et al*, 1993). Therapeutics, which increase cholinergic activity might be able to antagonise the EC lesion induced deficits.

It has been suggested that animals with lesions of the entorhinal area might be used as a deficit model for investigating cognitive deficits observed in neurodegenerative disorders such as Alzheimer's disease (Eijkenboom *et al*; 2000). In order to validate this model pharmacologically the effects the cognition enhancer Donepezil, which is used as therapeutic to treat Alzheimer's patients (Matthews *et al*, 2000;Giacobini, 2000), and Metrifonate, a compound that has successfully completed phase III clinical trials (Raskind *et al*, 1999;Gelina *et al*, 2000) were tested for their effect on Morris water escape performance of EC-lesioned rats.

Metrifonate is a cholinesterase inhibitors designed to increase the deficient acetylcholine levels in the brains of Alzheimer patients. These compounds, known as second generation cholinesterase inhibitors are long lasting, safe and well tolerated. They aim to restore levels of endogenous acetylcholine in the synaptic cleft by preventing its enzymatic breakdown by acetylcholinesterase. This study investigates whether these compounds are able to antagonise the EC lesion induced deficits.

Procedure

Material and Methods: Bilateral entorhinal cortex lesions

Surgery: All rats were anaesthetised with a solution (6 mg/kg) of 1.2 ml Ketavet (100 mg/ml^{-1} ketamin), 0.8 ml Rompun (2% xylacin) and 8.0 ml 0.9% NaCl and placed in a Sembach stereotaxic apparatus (Sembach Laborgeräte, Ratingen Rheinland, Germany). The dorsal skull was exposed and two holes were drilled above the target positions. With help of a stainless steel needle (Hamilton p/n: 80427/00 type 2; inner diameter 0.13 mm; outer diameter 0.47 mm) 10 mg/ml^{-1} ibotenic acid (molecular weight: 158.1; 95% purity; Sigma) solved in 0.9% NaCl (application volume: 0.5 µl) was injected bilaterally into each of three sites of the EC (injection speed 0.5 µl/min^{-1}; see also Eijkenboom *et al*; 2000). The co-ordinates are summarised in Table 1. The injection needle was left in place for 2 min after injection. The same procedure was followed for the sham control group, which received injections of the solvent (0.9% NaCl) only. The animals recovered well after surgery. No differences in gross behaviour were observed. The animals were tested in the Morris water escape task after a recovery period of one week.

	Co-ordinates of lesion sites		
	First set	**Second set**	**Third set**
Anterior-posterior	+ 1.0 mm	+ 1.2 mm	+ 1.5 mm
Lateral	± 6.8 mm	± 5.8 mm	± 6.6 mm
Dorso-ventral	+ 3.1 mm	+ 2.7 mm	+ 2.4 mm

Table 1: Stereotaxic co-ordinates used (according to Paxinos and Watson, 1986) to induce entorhinal cortex lesions by injections of ibotenic acid. The co-ordinates are with respect to the intra-aural line, the incisor bar was set at 3.3 mm above intra-aural line, and the injection needle was lowered at an angle of 15° in a mediolateral orientation

Morris water escape task

Apparatus: Morris water escape performance was assessed in a water tank which consisted of a circular grey tub with a slightly sloping wall (Material: polyethylene; inner dimensions: diameter at top 153 cm, diameter at bottom 143 cm, depth 63 cm), filled with 43.5 cm of clear tap water at a temperature of approximately 22°C. The escape platform consisted of a grey polyethylene cylinder (diameter 10.8 cm), submerged 1.5 cm below the surface of the water. In this version of the test the water was not made opaque because the grey escape platform was virtually invisible in the grey tank. The water tank was situated in a room illuminated by white fluorescent tubes. Abundant extra-maze cues were provided by the furniture in the room, including desks, computer equipment, a second water tank, the presence of the experimenter, and by a radio on a shelf that was playing softly. All testing was done between 9:00 and 15:00. A video camera, mounted in the centre above the circular pool, provided a picture of the pool on a TV-monitor. The movements of the rat were registered automatically by a video-tracking system (EthoVision®, Noldus Information Technology, Wageningen, The Netherlands) and stored in an MS-DOS compatible microcomputer.

One week after surgery the rats were tested in the Morris water escape task with four trials per session on five successive days. The test compound or vehicle was injected intraperiteoneally, 30 minutes before each daily training session.

A trial was started by placing a rat into the pool, facing the wall of the tank. Each of four starting positions (north, east, south, and west) was used once in a series of four trials; their order was randomised. The escape platform was always in the same quadrant. A trial was terminated as soon as the rat had climbed onto the escape platform or when 90 seconds had elapsed, whichever event occurred first. A rat was allowed to stay on the platform for 30 seconds. Then it was taken from the platform and the next trial was started. If a rat did not find the platform within 90 seconds it was put on the platform by the experimenter and was allowed to stay there for 30 seconds. After completion of the fourth trial (on the fifth day after completion of the probe trial, see below), the rat was gently dried with crêpe paper and returned to its home cage. The animal was kept warm under an infrared bulb (Original Hanau Solilux, 150 W) fixed about 60 cm above the floor of the cage.

Probe trial: After the fourth trial of the fifth daily session, an additional trial was given as a probe trial: the platform was removed, and the time the rat spent in the four quadrants was measured for 30 seconds. In the probe trial, all rats started from the same start position, opposite to the quadrant where the escape platform had been positioned during acquisition. All data were collected by the automatic video tracking system EthoVision® (Noldus Information Technology, Wageningen, NL).

Statistical analyses

Acquisition: Four different measures were taken to evaluate the performance of the rats during acquisition training: escape latency, travelled distance, swimming speed, and distance to platform.

Escape latency is the time (s) taken to find and escape onto the submerged platform (Morris, 1984). Travelled distance (cm) is the total distance swum to find and escape onto the submerged platform (Morris, 1984). Swimming speed was calculated as travelled distance (cm) divided by escape latency (s). Distance to platform (cm) (Gallagher, Burwell & Burchinal, 1993) was calculated as the mean distance to the platform across all samples drawn by the video-tracking system between the start of a trial and the moment the rat climbed onto the platform.

The measures were averaged per rat within each session. Treatment effects on the acquisition of the water escape task were assessed with an analysis of variance (ANOVA; Cotton, 1998; Winer, 1971) with repeated measures over sessions. In addition, treatment effects in particular sessions were analysed by ANOVA, complemented by post hoc comparisons where appropriate.

Probe trial: Treatment effects of the time (s) in quadrants during the probe trial were assessed with a repeated measures ANOVA *over* quadrants (time in the quadrant north, east, south, and west are considered as levels of the repeated measures factor), complemented by ANOVAs on the swimming times *per* quadrant.

Experiment 1: The effects Metrifonate on spatial discrimination performance of entorhinal cortex lesioned rats

Animals: 47 male HsdWin: Wu rats, weighing between 266 and 357 grams were supplied by Harlan Winkelmann, Borchen, Germany. They were allowed to adapt to our animal facilities for one to two weeks before surgery. The animals were assigned randomly to their respective treatment conditions in two parallel experiments, in each of these two experiments; half of the animals per treatment condition were tested.

Drug Administration: Metrifonate was administered p.o., solved in Na-citrate buffer, pH 5.5, at an application volume of 2 ml/kg^{-1} body weight at concentrations 10 or, 30mg/kg, 30 min before each of the five training sessions.

Results

Acquisition

Escape latency (see Fig. 2, *upper left panel*): Averaged over the five successive acquisition sessions, the time to escape onto the submerged platform was affected by the treatments (General mean: $F_{3,43} = 19.37$, $p < 0.01$). Post hoc comparisons revealed the following: The EC-lesioned control group needed, on average, more time to locate the platform than the sham-lesioned control group. Treatment with

30 mg/kg^{-1} was able to improve the EC lesion induced deficit; this group of rats needed more time than the sham-lesioned control group, but less time than the EC-lesioned control group to find the submerged platform. The performance of the group treated with 10 mg/kg^{-1} Metrifonate did not differ from the EC-lesioned control group and the group treated with 30 mg/kg^{-1} Metrifonate. All groups reduced the time to escape onto the platform in the course of training (Sessions: $F_{4,172} = 45.11$, $p < 0.01$) to a similar degree (Sessions by Treatment interaction: $F_{12,172} = 1.18$, n.s.).

Distance travelled (see Fig. 2, upper centre panel): The average distance travelled was affected by the treatments (General mean: $F_{3,43} = 14.55$, $p < 0.01$). Post-hoc comparisons confirmed that the EC lesion affected the performance; this deficit was not ameliorated by treatment with Metrifonate. In the course of training, all groups reduced the swim path (Sessions: $F_{4,172} = 40.02$, $p < 0.01$). The speed of learning was not affected by Metrifonate treatment (Sessions by Treatment interaction: $F_{12,172} = 1.44$, n.s.).

Swimming speed (see Fig. 2, upper right panel): The average swimming speed was not affected by the treatments (General mean: $F_{3,43} = 0.33$, n.s.). The swimming speeds slightly changed across sessions (Sessions: $F_{4,172} = 4.18$, $p < 0.01$), but these changes were similar for the groups (Sessions by Treatment interaction: $F_{12,172} = 0.78$, n.s.).

Distance to platform (see Fig. 2, lower left panel): Treatments affected the average distance to platform (General mean: $F_{3,43} = 14.65$, $p < 0.01$). Post hoc comparisons confirmed that the sham-lesioned control group swam, on average, at a shorter distance from the platform that did the EC-lesioned control group. The mean distance to platform of the EC-lesioned group of rats treated with 30 mg/kg^{-1} Metrifonate was shorter than that of the EC-lesioned control group, but longer than

that of the sham-lesioned control group, indicating that the drug-treatment partially antagonised the EC-lesion induced deficit. The group treated with 10 mg/kg^{-1} Metrifonate did not differ from the EC-lesioned controls. Distance to platform decreased across training sessions (Sessions: $F_{4,172} = 49.45$, $p < 0.01$), and the decrease was different for the groups of rats (Sessions by Treatment interaction: $F_{12,172} = 1,98$, $p < 0.05$). The variation in the decrease of the distance to platform across sessions was nearly exclusively covered by the linear trend component (97%). As confirmed by post-hoc comparisons, the group treated with 30 mg/kg^{-1} Metrifonate showed a steeper decrease of the distance swum in the course of training than the sham-lesioned and the EC-lesioned control groups, indicating that they learned faster than the other groups of rats. However, the shape of their learning curve did not differ from that of the group treated with 10 mg/kg^{-1} Metrifonate which might indicate that this dose also tended to improve speed of learning.

Probe trial

Time spent in quadrants (see Fig. 2, lower right panel): The time spent in the four quadrants was different during the probe trial (Quadrants: $F_{3,129} = 8.45$, $p < 0.01$). The treatments affected the bias for particular quadrants (Quadrants by Treatment interaction: $F_{9,129} = 2.37$, $p < 0.05$). Time spent in quadrant West, i.e. the quadrant where the platform had been positioned during training, was affected by the treatments ($F_{3,43} = 5.37$, $p < 0.01$). Post hoc comparisons revealed that the sham-lesioned groups spent more time in this quadrant than any of the other groups, which did not differ from one another. The EC-lesion apparently disrupted the bias for the training quadrant. This effect was not antagonised by treatment with Metrifonate.

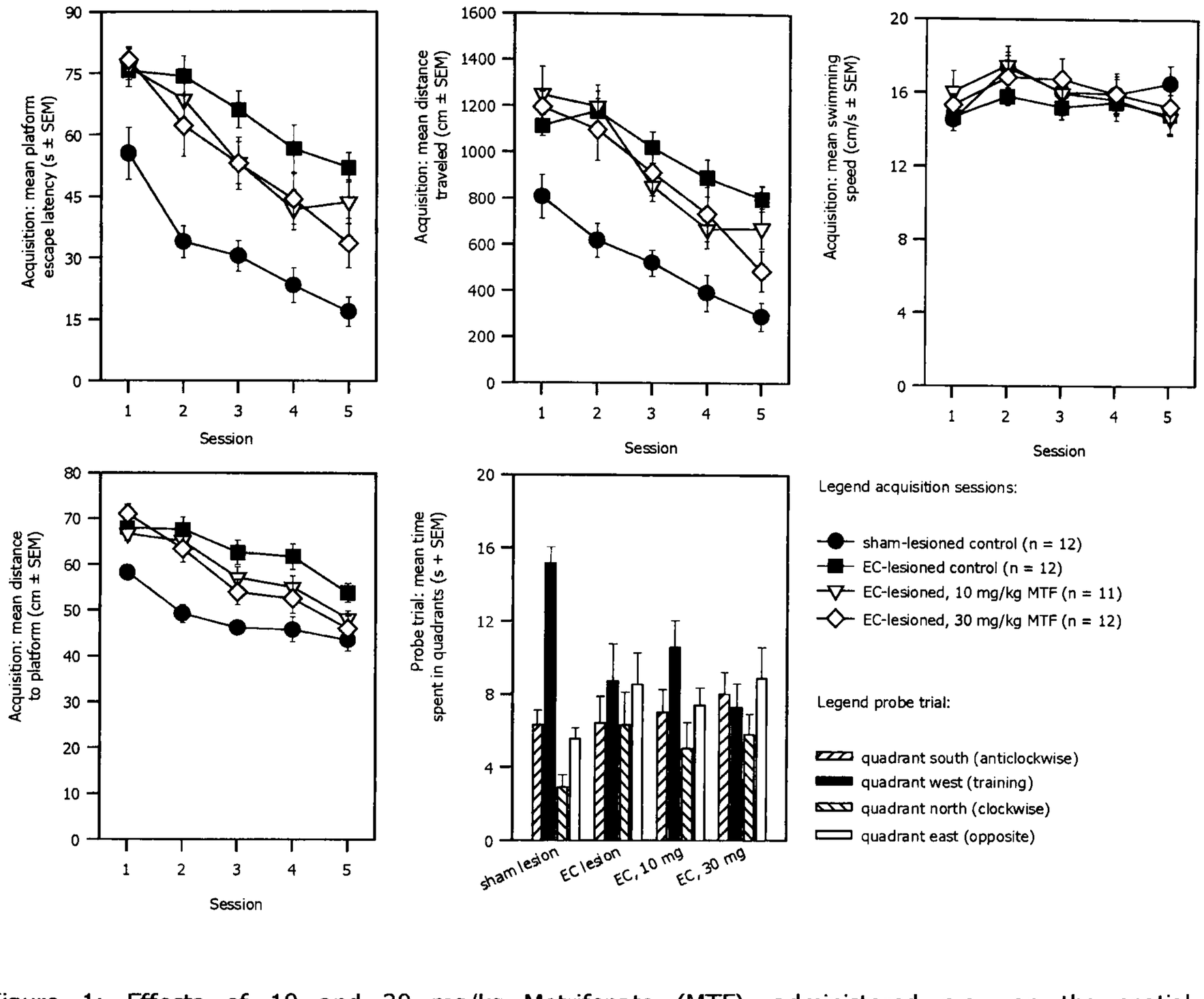

Figure 1: Effects of 10 and 30 mg/kg Metrifonate (MTF), administered p.o., on the spatial discrimination performance of male Wistar rats with bilateral lesions of the entorhinal cortex (EC) region. The escape latency (*upper left*), distance travelled (*upper centre*), swimming speed (*upper right*), and distance to platform (*lower left*) during acquisition are depicted. In addition, the time (*lower right*) spent in the four quadrants of the pool during the probe trial is shown. All data are presented as means and standard errors of the means (SEMs).

Discussion

Paradigms employing brain lesions, typically in rats, have been used to study the neural basis of cognitive dysfunctions to evaluate potential therapeutic approaches. In the present study, ibotenic acid lesioned rats were used to assess the effects of metrifonate, a ChE-inhibitor. Ibotenic acid destroys cell bodies without concurrent damage to fibres of passage, which can occur by mechanical lesions.

Early lesion models of AD focused primarily on the nucleus basalis of Meynert and the medial septum. Deficits were reported in passive avoidance retention, spatial swim maze learning and other reference memory tasks, as well as various working memory tasks (Dunnett et al, 1991;Olton et al, 1987). However, as previously

discussed the initial stages of AD are characterised by a neuropathological changes in the entorhinal cortex (Braak and Braak, 1991; Hyman et al, 1986; Miwa & Ueki, 1996). Rats with excitotoxic lesion of the EC have previously been shown to impair acquisition of both passive and active avoidance learning and positive reinforcement operant learning with no increase in locomotor activity and sensitivity (Miwa et al, 1996; Ueki et al, 1996) as well as spatial ability in the water maze task (Schenk and Morris, 1985).

In this study, spatial discrimination deficits, induced by bilateral lesions aimed at the entorhinal cortex region, could, at least partially, be antagonised by treatment with cholinesterase inhibitors (ChE-I). In the present study, Metrifonate was tested. The compound improved the level of performance compared with that of the EC-lesioned control groups (Metrifonate at 30 mg/kg^{-1}). No changes in the swimming pattern were observed in animals with EC lesions (i.e. they did not exhibit wall hugging or thigmotaxic behaviour, see chapter 6) and with no overall changes in the swimming speed suggested that the EC lesioned animals swam further around the maze indicating an increase in exploratory activity to escape to the platform.

Conclusions

These data support the notion that the deficiency model of the bilaterally EC-lesioned rat is sensitive to the effects of putative cognition enhancing compounds, and are in line with reports that compounds modulating the AMPA receptor (Aniracetam; Zajaczkowski and Danysz, 1997) or the NMDA receptor (memantine; Zajaczkowski *et al*, 1996), or administration of the neuroprotective compound ganglioside (Ramirez *et al*, 1991; Ramirez *et al*, 1998) improve cognitive functions in EC-lesioned rats. Bilateral EC-lesioned rats model some of the pathological and behavioural changes observed in Alzheimer patients, such as degenerations in the EC, which occur very early during the development of the Alzheimer pathology, concomitant dysfunctions of hippocampal circuits, and spatial discrimination deficits. The present experiments indicate that under conditions of pathological impairment of brain structures such as entorhinal cortex lesion, ChE-Is might produce beneficial effects on learning and memory.

Effects of Metrifonate on the Timing Behaviour in the Rat.

Abstract

The peak interval (PI) procedure is an operant conditioning schedule that has been suggested as a useful paradigm for assessing and characterising putative cognition enhancers. These compounds aim to reduce cognitive dysfunction prominent in conditions such as Alzheimer's disease.

There is ample evidence that cholinergic deficits have a role in cognitive impairments. We tested metrifonate in a PI 20s task. This compound is a cholinesterase inhibitor that has been shown to enhance cognition in various animal models. Results obtained with this temporal procedure are interpreted according to the Scalar Expectancy theory (SET).

Cognition enhancers are expected to shift the peak time to the left, reflecting a change in time estimation, perhaps caused by an increase in the speed of information processing. In the PI procedure a single administration of metrifonate shifted the peak time to the right, whereas sub-chronic administration of metrifonate had no effect on time estimation. Scopolamine produced a reduction in the spread possibly indicating a more accurate perception of the time of reinforcement and an increase in the run rate indicating an increased motivation to the task, however no other timing effects were observed. Therefore we are not able to conclude as to the sensitivity of this paradigm for assessing cognition enhancers with a procholinergic mode of action.

Introduction

Alzheimer's disease (AD) is a chronic neurodegenerative disorder characterised by a progressive decline in cognitive function, including loss of memory and cognitive function and behaviour. The causes of the disease are as yet unknown, however central cholinergic hypofunction is thought to be a component of the disease. Several lines of evidence support this notion that a loss of cholinergic function contributes the cognitive deficits seen in AD (Bierer, *et al*, 1995). Acetylcholine (ACh) synthesis in nerve terminals is decreased due to a reduction in choline acetyltransferase (ChAT) activity. The brains of AD patients characteristically show a dramatic loss of neurones and synapses in many areas, particularly in the basal forebrain, amygdala, hippocampus and cerebral cortex (Bartus, Dean, Pontecorvo, and Flicker, 1985).

Initial approaches to AD were to enhance cholinergic function. Cholinomimetic agents can augment cholinergic transmission either directly or indirectly. The most successful of these strategies at this time is to increase the amount of Ach by reducing its degradation by cholinesterase inhibitors (ChEI's). Metrifonate donepezil and rivastigmine are three such ChEI's currently available.

In rats, lesions of the nucleus basalis magnocellularis (nbm) - an area of the basal forebrain that has significant projections to the frontal cortex, and where substantial degeneration is found in patients with Alzheimer's disease - produce an over estimation in the remembered time of reinforcement. From this, it can be suggested that AD patients could have a defective accuracy in time estimation (Nichelli, Venneri, Molinari, Tavani, & Grafman 1993). This observation leads us to think that cholinesterase inhibitors for the treatment in AD could be assessed using a timing behaviour model.

Timing behaviour is thought to be a cognitive function of the brain in terms of a subjects' ability to control the way in which stimulus information is integrated over time. In the present study the discrete trial peak interval procedure was used. This operant conditioning schedule is an extension of the fixed interval (FI) procedure in which a lever is presented and the animal is free to respond at any time, but only the first response after a fixed duration (e.g. 20 seconds) is reinforced. In the peak interval (PI) procedure some trials are identical to the FI procedure where others consist of the lever presented for a time that goes beyond the fixed duration (e.g. 50 seconds in our experiments, and typically at least twice plus a random duration of the fixed interval) and no reinforcement is given.

Data is derived from the non-reinforced trials, or PI trials. When averaging across trials and subjects the response distribution appears to be a Gaussian like function with a slight positive skew (Catania, 1970; Roberts, 1981; Church, Miller, Meck & Gibbon, 1991). The peak of the distribution occurs near the time that food is maximally expected (and usually granted) and is called the peak time (PT). The response rate at the PT is the peak rate (PR; see figure 1, left panel). PT and PR are independent values (Roberts, 1981), so that timing can be compared under conditions in which the absolute rate of responses varies.

Response distribution on individual trials is rather different and can be characterised by a three- phase process called a low-high-low or break-run-break pattern of responding (to follow the terminology suggested by Schneider, 1969). The animal often responds at a low rate at the beginning of the trial, then begins responding at fast and steady rate and finally returns to a low rate. Performance on each of these individual trials can be further investigated by measuring the time at the beginning of high responding (S1), the time on stopping of high responding (S2), the middle of this high responding rate (M, middle = (S1+S2)/2) and the duration of responding (D, spread = S2-S1; see figure 1, right panel)

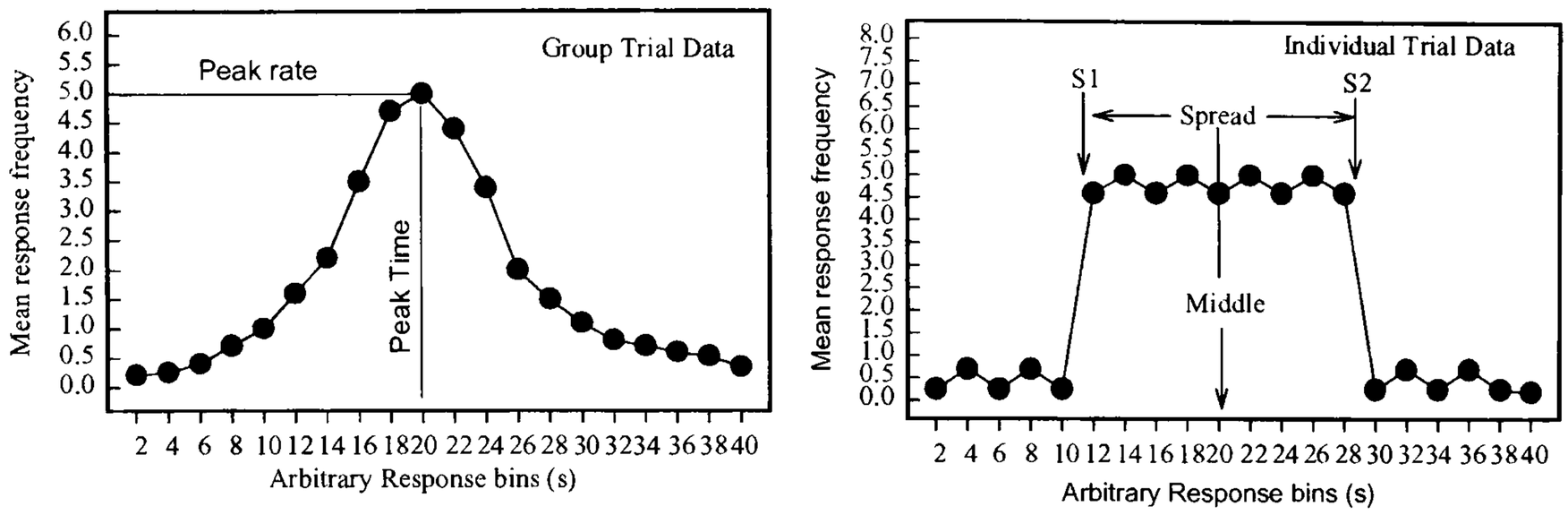

Figure 1: Group trial data*: (left panel).* The response distribution obtained is Gaussian with a slight positive skew Two major read outs are considered: 1: the peak time (PT), time at which the food is maximally expected and 2: the peak rate (PR), the maximum response rate at this time. Individual trial data: *(Right panel)* The response distribution obtained follows a low-high pattern of responding. Four read outs are considered: S1**:** the start time, time at which the animal begins to respond at a high rate, S2: the stop time, time at which the animal stops to respond at a high rate, the spread: time during which the animal responds at a high rate, the middle: time halfway through the spread

Performance on each of these individual trials can be further investigated by measuring the time at the beginning of high responding (S1), the time on stopping of high responding (S2), the middle of this high responding rate (M, middle = (S1+S2)/2) and the duration of responding (D, spread = S2-S1; see figure 1, right panel

Today, the most challenging theoretical framework explaining timing behaviours is the Scalar Expectancy Theory (SET, Gibbon *et al*, 1984) that involves an internal clock as well as memory and decision processes. According to SET, on presentation of a signal, the internal clock emits pulses that are switched into an accumulator. The accumulated pulses (n) are then compared to a representation of expected time of reinforcement sampled from the reference memory (n*). The representation of actual time and the representation of the usually reinforced time are compared at the decision level. When the two representations are close enough, that is when the discrepancy between them lies below a certain threshold, a response is made (more precisely, the probability to respond fall over from low to high). If a response is reinforced, the value (n) accumulated in the accumulator is transferred to the distribution of values kept in the reference memory (Gibbon *et al*, 1984).

The 'internal' clock is used for measuring the speed in which information is integrated and this appears to be linked to dopamine function in the basal ganglia and the memory storage process used for the representation of the duration's of prior events

appears to be linked to acetylcholine function in the frontal cortex. These two systems appear to be linked by frontal striatal loops (Meck, 1996).

The first aim of the present study was to determine the effects of chronic metrifonate on the peak interval procedure. The hypothesis is that this cholinesterase inhibitor administration schedule facilitates the acquisition of time estimation using the PI20s procedure. The second aim of our study was to determine the effect of a single administration of the ChEI, metrifonate on the peak time and peak rate on the 20s peak interval procedure in well trained rats. The effects of scopolamine was also assessed to observe the effects of an anti cholinergic compound on the peak interval procedure. The usability of these methods to assess putative cognition enhancers is discussed.

Procedure

Apparatus: The rats were trained in 10 similar standard lever boxes (manufactured by Electronic and Computer Engineering Dept. & Mechanical Engineering Dept., University of Nijmegen, The Netherlands; inner dimensions: 525 mm wide, 270 mm deep, 280 mm high) The front wall contained a food tray (34 mm deep) through which pellets (BioServ, USA. 45 mg) could be delivered and two retractable stainless steel levers (40 mm wide, projecting 20 mm into the Skinnerbox), were located at a distance of 48 mm from both sides of the food tray. The chamber was fitted with white and red house lights on the roof, and was enclosed in a sound-attenuating chest. Each chamber was equipped with a fan for ventilation (*See figure* 2) An Apple Power Macintosh controlled the experiments and collected the data.

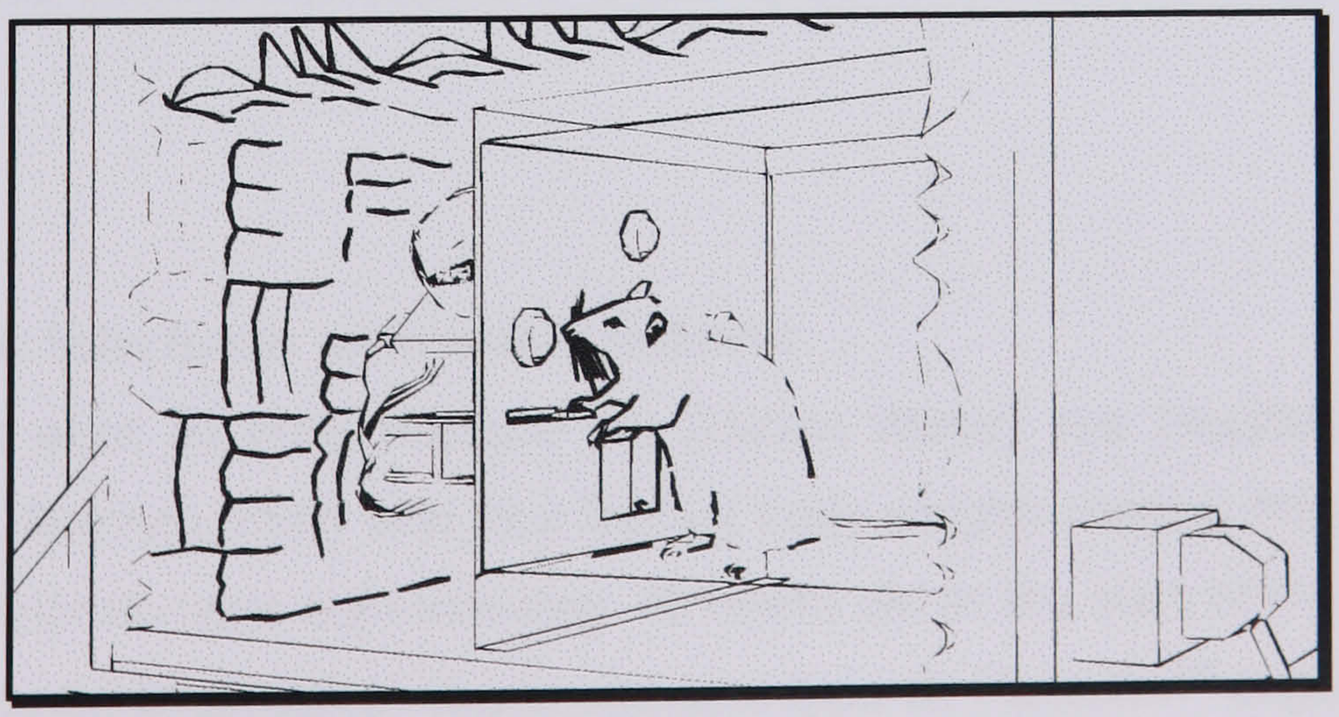

Figure 2: Diagram of the Skinner box

Methods

The rats were deprived to 85-90% of their free feeding weight and after each experimental session an adjusted ration of food (Ssniff Food pellets) was given to maintain this deprivation level.

Pre-Training: The rats were magazine trained in the Skinner box for 30 minutes until they had consumed at least twenty pellets for two consecutive days. The pellets were randomly supplied at inter-trial interval (ITI) ranging from 20 to 100 seconds. During this phase the levers were retracted. During the continuous reinforcement training two levers (left and right) were presented alternatively. After pressing the lever, the lever was retracted and a 45 mg food pellet was delivered. An ITI interval of 10 seconds spaced the visit of the food tray and the presentation of the next trial. The start of each trial was signalled by the onset of the white house lights. The training ended after reaching a criterion of 50 lever presses in 30 minutes in two consecutive days. This was followed by FI training that consisted of a lever being presented and only the first lever press following a fixed amount of time is reinforced with a pellet. The FI training started with fixed interval schedule of 5 seconds (FI 5) where an ITI of 20 seconds was inserted between successive trials. The lever, left or right, was counterbalanced over the rats and remained the same during the experiment. When the rats obtained 85% or more of the maximum number of pellets in a session the fixed interval increased to 10 seconds (FI 10) and then onto 20 seconds (FI 20).

Peak interval (PI) Training. Once a steady rate of responding had established PIs were introduced. On random half of the trials, the rats were given fixed intervals. On the remaining trials, food was not primed at 20 seconds after trial onset. After 50 seconds the trial ended without deliverance of a pellet, the lever was retracted, and the ITI started.

Drug testing

Experiment 1: Sub-chronic metrifonate treatment

The subjects were 30 male HsdCpd: Wu (Harlan Wistar) rats (supplied by Harlan-Winkelmann, Borchen, FRG) with free-feeding body weights ranging from 220 to 250 grams. The animals were approximately 10 weeks old at the start of the experiment. Rats were housed in groups of two in standard type III Makrolon^{TM} cages, water was continuously available, with a 12:12 hour light/ dark cycle, lights on at 07.00 am.

After 2-3 weeks of pre-training, drug administration began on day one of PI20" training and continued daily, 5 days per week for approximately 36 days. Metrifonate, 10 mg/kg (n=7), 30 mg/kg (n=8), 50 mg/kg (n=8) or vehicle [sodium citrate buffer, pH. 5.5. (N=7)] was administered orally through a syringe (p.o) 30 minutes before the start of the peak-interval session that lasted 30 minutes. The administration volume was 5ml/kg

Experiment 2: Acute metrifonate or scopolamine treatment in rats trained to a stable performance.

The subjects were 45 male Hsdcpd: Wu (Harlan Wistar) rats (supplied by Harlan-Winklemann, Borchen) with free-feeding body weights ranging 220- 250 grams from 10 weeks old at the start of the experiment. Housing conditions and food deprivation were the same as for Experiment 1. After 2-3 weeks of pre-training, PI training continued for 40 sessions, once daily for five days per week before any drug administration. See Table 1 for drug administration protocol.

Table 1: Drug administration protocol for metrifonate, rivastigmine, donepezil and scopolamine in well trained rats in the PI20s procedure.

Metrifonate	**Scopolamine**
Session 40	Session 70
3 mg/kg, 10 mg/kg 30 mg/kg 60 mg/kg or vehicle (sodium citrate buffer, pH, 5.5)	0.1 mg/kg, 0.3 mg/kg, 1mg/kg, 3 mg/kg (n=9) or vehicle [sodium Chloride 0.9%)

All groups are N=9 (except 60 mg/kg metrifonate where N = 8). All compounds with the exception of scopolamine were administered orally 30 minutes prior to the peak interval sessions. Scopolamine was administered interperitoneally (i.p)

Data Analysis: In all drug sessions, results were only taken from the peak trials. The peak time was determined as follows. The number of responses in each 4-second interval (1-4, 2-5, 3-6 etc.) was counted and the maximum number of responses was identified. The median time for each of these intervals was found. If there was only one such interval, its median was defined as the

peak time. If there were several such intervals the median of their medians was defined as the peak time (Meck, Komeily-Zadah & Church, 1984).

For each peak trial there was a measure of the time that the rat started to respond at a high rate (S1) and the time at which it stopped at the high rate (S2), These times were determined by an exhaustive search of all the possible locations to maximise the difference between the high rate and the low rates, weighted the relative time in the high and low states. On each trial the index that was maximised was t1*(r-r1)+t2*(r2-r)+t3*(r-r3), where t1, t2 and t3 were the times until S1, the time between S1 and S2 and the time from S2 until the end of the trial. r1, r2 and r3 were the mean response rates corresponding to these times and r is the overall mean response rate. The values reported were for all peak trials on which S1 was less or equal to the time of reinforcement (T*), which indicates good starts and S2 was greater than T* indicating good stops. Determined from S1 and S2 was the middle of this time interval (M=(S1+S2)/2) and the spread of this time interval (D=S2-S1) The run rate is the mean response rate during the spread of the high responding period (Church, Meck & Gibbon, 1994 were the first to use this formula,

Data presented as start, stop, spread, middle [respectively S1, S2, D and M], peak time and run rate in this report are means ± standard errors of the means. In experiment 1, the effect of sub-chronic metrifonate treatment on the timing behaviour in rats was analysed in blocks of 4 days over the period of 36 days of treatment (Metrifonate Vs vehicle) and this was analysed by TREATMENT*BLOCKS ANOVA with repeated measures on the last factor. This was further assessed by a one factorial ANOVA on orthogonal trend components supplemented with Fisher's least significant difference (LSD) post hoc comparisons. In experiment 2, treatment effects were analysed by difference scores (Delta) between pre-treatment and treatment sessions (day before drug administration and day of drug administration) and this was supplemented with Fisher's least significant difference (LSD) post hoc comparisons, supplemented by Students t-test to test whether the treatment groups deviated from zero.

Results

Experiment 1: Sub-chronic metrifonate treatment (see figure 2)

S1 (*See fig 2, panel A*): Averaged across the nine blocks of 4 sessions (GENERAL MEAN: $F_{3,22}$ = 0.59, n.s) metrifonate treatment had no effect on S1. S1 decreased in the course of training (BLOCKS: $F_{8,176}$ = 24.19, $p<0.01$), however this decrease was similar for the treatment groups (BLOCKS by TREATMENT interaction: $F_{24,176}$ =0.88, n.s)

S2 (*See fig 2, Panel B*): Metrifonate treatment had no effect on S2 when the average across the nine blocks of 4 sessions was considered (GENERAL MEAN: $F_{3,22}$ = 1.24, n.s). S2 was decreased in the course of training (BLOCKS: $F_{8,176}$ = 8.04, $p<0.01$), similarly for the treatment groups (BLOCKS by TREATMENT interaction: $F_{24,176}$ =0.92, n.s)

Middle (*See fig 2, Panel C*): Metrifonate treatment had no effect on the middle, averaged across the nine blocks of 4 sessions (GENERAL MEAN: $F_{3,22}$ = 0.87, n.s). The middle was decreased in the course of training (BLOCKS: $F_{8,176}$ = 16.07, $p<0.01$), this decrease however was similar in all groups (BLOCKS by TREATMENT interaction: $F_{24,176}$ =0.75, n.s)

Spread (*See fig 2, Panel D*): The average spread across the nine blocks of 4 sessions (GENERAL MEAN: $F_{3,22}$ = 0.23, n.s) was not affected by metrifonate treatment. The spread was decreased in the course of training (BLOCKS: $F_{8,176}$ = 7.52, $p<0.01$), at a similar rate in all the treatment groups (BLOCKS by TREATMENT interaction: $F_{24,176}$ =1.18, n.s)

Run Rate (*See fig 2, Panel E*): Averaged across all sessions (GENERAL MEAN: $F_{3,22}$ = 11.16 $p<0.01$) metrifonate treatment decreased the run rate. Post hoc comparisons revealed that the 10, 30 and 50 mg/kg differed from the vehicle treated group. There was an increase in the run rate during the course of training (BLOCKS: $F_{8,176}$ = 59.66, $p<0.01$), and this was affected by metrifonate treatment. (BLOCKS by TREATMENT interaction: $F_{24,176}$ = 48.47, $p<0.01$). Subsequent orthogonal trend components showed that the change in run rate can be described by a linear ($F_{3,22}$ = 7.27, $p<0.001$), quadratic ($F_{3,22}$ = 29.86, $p<0.001$) and a cubic ($F_{3,22}$ = 66.89,

$p<0.001$) trend, which covered 27.62%, 4.16% and 13.64% of the variance respectively. T- tests showed that all groups were covered by the linear trend (vehicle: $t_6 = 10.89$, $p< 0.001$; 10 mg/kg: $t_6 = 2.92$, $p< 0.05$.; 30 mg/kg: $t_5 = 5.62$, $p< 0.05$.; 50 mg/kg: $t_5 = 3.67$, $p< 0.05$). For the quadratic trend only the vehicle group was covered ($t_6 = -9.19$, $p< 0.001$). 10, 30 and 50 mg/kg could not show a second order trend ($t_6 = 0.78$, n.s.; $t_5 = 0.05$, n.s.; $t_5 = -0.44$, n.s. respectively). For the cubic trend only the vehicle group was covered ($t_6 = -12.47$, $p< 0.001$). 10, 30 and 50 mg/kg could not show a third order trend ($t_6 = 0.79$, n.s.; $t_5 = -0.41$, n.s.; $t_5 = -0.66$, n.s. respectively).

Peak time (*See fig 2, Panel F*): The average Peak time across the nine blocks of 4 sessions (GENERAL MEAN: $F_{3,22} = 1.62$, n.s) was not affected by metrifonate treatment. The peak time was decreased in the course of training (BLOCKS: $F_{8,176} = 9.53$, $p<0.01$), however this decrease was similar for all groups. (BLOCKS by TREATMENT interaction: $F_{24,176} = 0.75$, n.s).

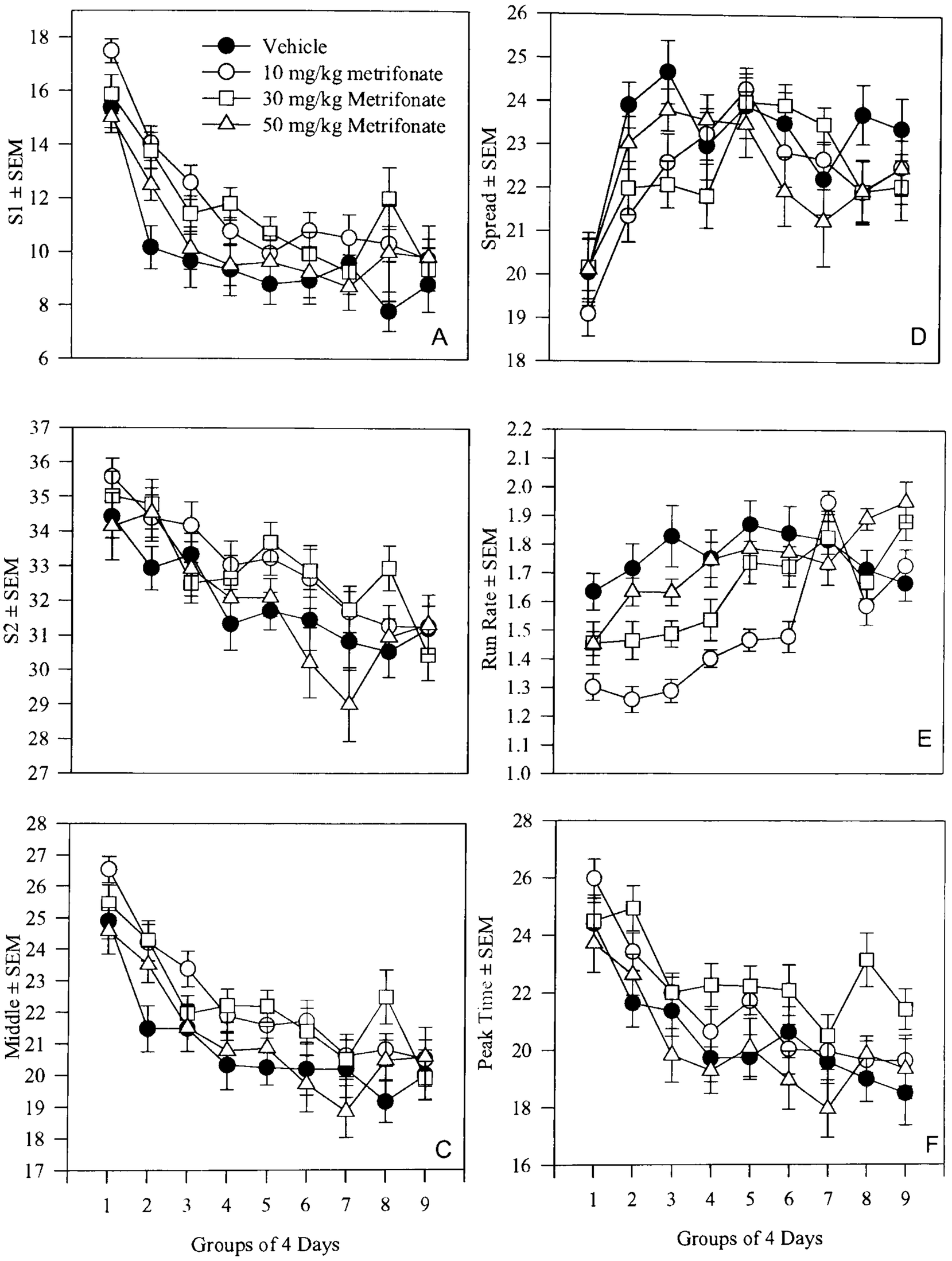

Figure 3: Effects of sub-chronic metrifonate (10, 30 and 50 mg/kg) on the peak interval procedure in Harlan Wistar rats over a period of 36 days. The effects on S1, (*Upper left*) S2, *(Middle Left*), Middle (*Lower left),* Spread *(Upper Right)* PR *(Middle right)* and PT *(Lower right)* were observed All results are ± SEM and N=7-8.

Experiment 2: Acute metrifonate treatment on well trained rats. (Figure 4)

S1: Treatment with metrifonate affected the difference score of S1 between the pre-treatment and the treatment sessions ($F_{4,38}$ =7.06, $p<0.05$). Post-hoc analysis confirmed that metrifonate, 3, 30 and 60 mg/kg increased S1 compared with vehicle treated group. This was confirmed by t-tests per treatment groups: There was no difference between the two sessions in the controls (t_8 = 2.01, n.s.) whereas the different scores differed from zero in the 3 mg/kg group (t_8 = -5.17, $p<0.01$) and the 60 mg/kg group (t_7 = -2.96, $p<0.05$). The 30 mg/kg did not differ from zero (t_8 = -1.45, n.s).

S2: Treatment with metrifonate affected the difference score of S2 between the pre-treatment and the treatment sessions ($F_{4,38}$ = 6.88, $p<0.01$). Post-hoc analysis confirmed that metrifonate, 3 and 60 mg/kg increased S2 compared with vehicle treated group. This was confirmed by t-tests per treatment group: There was no difference between the two sessions in the controls (t_8 = 1.29, n.s.) whereas the difference scores differed from zero in the 60 mg/kg group (t_8 = -4.55, $p<0.01$).

Spread: The spread was not affected by metrifonate treatment ($F_{4,38}$ =1.01, n.s).

Middle: Treatment with metrifonate affected the difference score of the middle between the pre-treatment and the treatment sessions ($F_{4,38}$ = 2.77, $p<0.05$). Post-hoc analysis confirmed that metrifonate, 60 mg/kg increased the middle compared with vehicle treated group. This was confirmed by t-tests per treatment group: There was no difference between the two sessions in the controls (t_8 = -0.11, n.s.) whereas the difference scores differed from zero in the 3 mg/kg group (t_8 = -6.26, $p<0.01$) and the 60 mg/kg group (t_7 = -4.04, $p<0.01$).

The run rate was not affected by metrifonate treatment ($F_{4,38}$ =1.83, n.s).

Peak time: Treatment with metrifonate affected the difference score of the peak time between the pre-treatment and the treatment sessions ($F_{4,38}$ =5.90, $p<0.01$). Post-hoc analysis confirmed that metrifonate, 60 mg/kg increased the peak time compared with

vehicle treated group. This was confirmed by t-tests per treatment group: There was no difference between the two sessions in the controls (t_8 = 0.29, n.s.) whereas the difference scores differed from zero in the 60 mg/kg group (t_7 = 3.48, $p<0.05$).

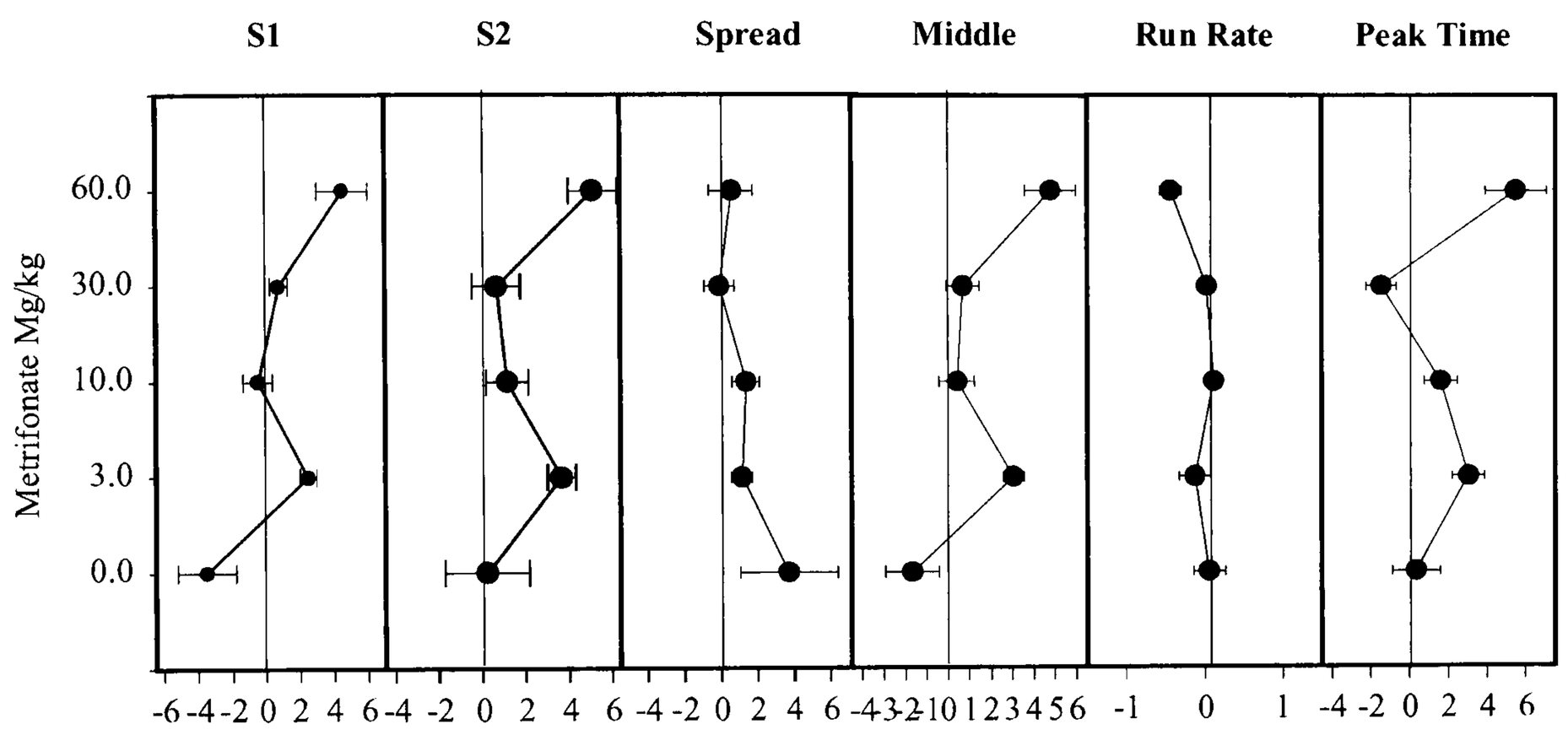

Figure 4: Differences in group trial (PT and Run Rate) and individual trial measures (S1, Middle, S2, Spread) between the day of treatment and the day before treatment when testing single administration of metrifonate in the PI procedure. Metrifonate (3, 10, 30 and 60 mg/kg) was injected p.o. in an application volume of 10 ml/kg. All results are ± SEM and N=9, except in the 60 mg/kg group, where N=8.

Scopolamine treatment on well trained rats (Figure 5).

Animals were removed from analysis when S1 was not less or equal to 20 seconds and/or S2 was not greater than 20 seconds.

S1 showed no effect of scopolamine treatment, because performance was not different between the pre-treatment and the treatment sessions ($F_{4,30}$ = 1.54, n.s.).

S2 showed no effect of scopolamine treatment, because performance was not different between the pre-treatment and the treatment sessions ($F_{4,30}$ = 0.48, n.s.)

Treatment with scopolamine affected the difference score of the spread between the pre-treatment and the treatment sessions ($F_{4,38}$ = 4.09, $p<0.05$). Post-hoc analysis confirmed that scopolamine, 1 mg/kg decreased the spread compared with that of the vehicle treated group.

Scopolamine treatment did not affect the middle ($F_{4,30}$ = 0.14, n.s)

The difference score on the run rate was affected with scopolamine treatment as there was a change from the pre-treatment to the treatment session ($F_{4,30}$ = 5.26, $p<0.05$). Post hoc analysis confirmed that scopolamine 1 and 3 mg/kg increased the run rate compared with that of the vehicle treated group.

The peak time was similar in the pre-treatment to treatment sessions i.e. scopolamine treatment did not affect this measure ($F_{4,30}$ =0.25, n.s).

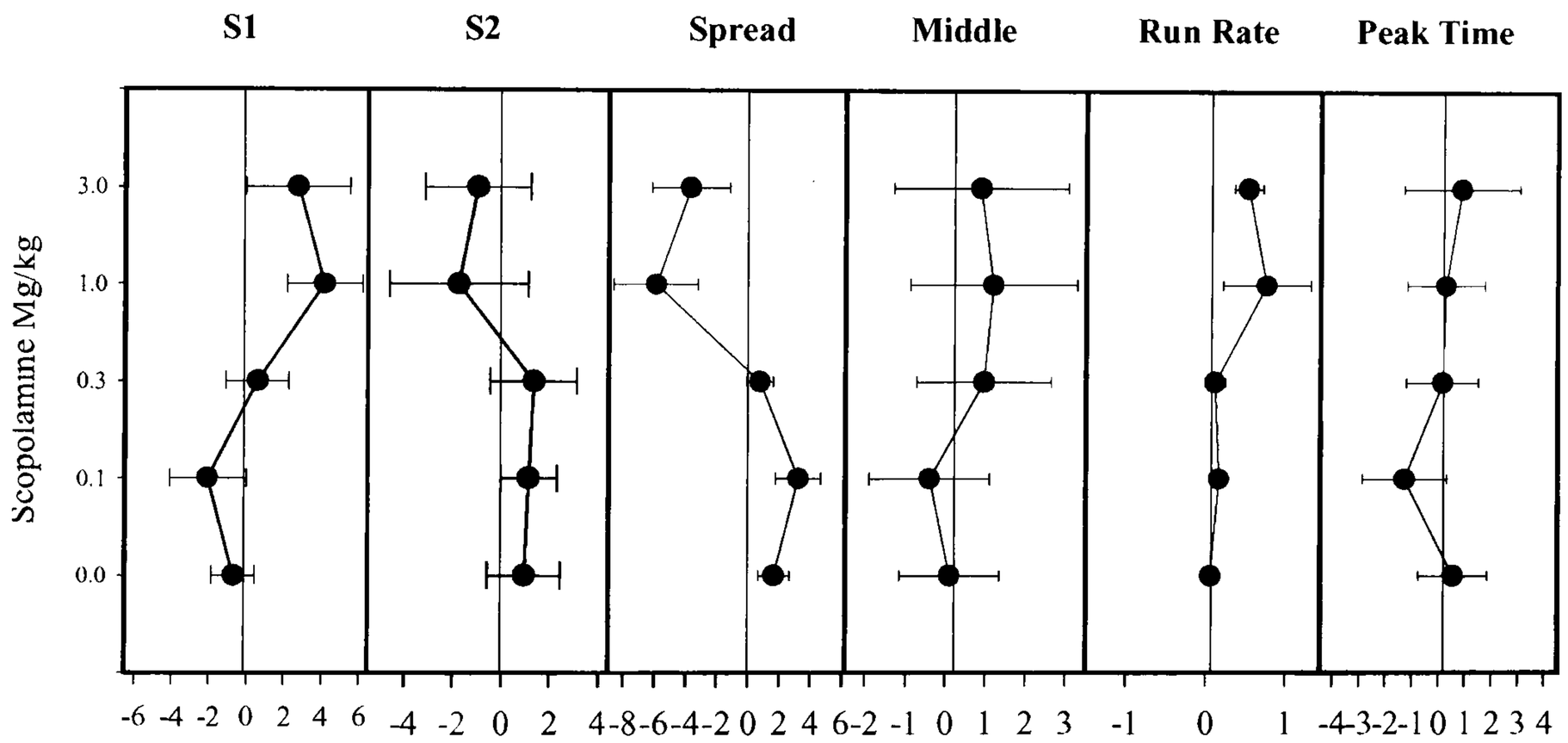

Figure 5: Differences in group trial (PT and Run rate) and individual trial measures (S1, Middle, S2, Spread) between the day of treatment and the day before treatment when testing single administration of scopolamine in the PI procedure. Scopolamine (0.1, 0.3, 1 and 3 mg/kg) was injected p.o. in an application volume of 10 ml/kg. All results are ± SEM

Discussion

Summary of Results

The present results are consistent with many previous studies with the PI procedure. Group trial data showed a Gaussian-like distribution (data not shown: Roberts, 1981; Meck and Church, 1987a; Meck and Church, 1987b), and individual trial data showed a low-high-low distribution of responding (Cheng & Westwood, 1993; Church *et al*, 1994).

Sub chronic metrifonate (10, 30 and 50 mg/kg) was given over a period of 36 days during PI20s training. Temporal discrimination acquisition was reflected by changes in

the Peak Time (PT), Spread and Middle. Metrifonate did not predominantly improve the acquisition of this task.

Acute metrifonate in well trained rats increased S1, S2, middle and peak time (60 mg/kg) and did not affect the spread and run rate. Scopolamine (1 mg/kg) produced a decrease in the spread and an increase in the run rate. No other effects were observed and especially no changes imply the middle or peak time. This point is important as it could be interpreted as an improvement of the performance as more lever presses are concentrated around the reinforced time.

Timing behaviour and the cholinergic system

It has previously been reported that, in the peak procedure, cholinergic compounds such as choline (Meck and Church, 1987a) and physostigmine (Meck and Church, 1987b) will shift the gaussian curve to the left indicating a change of the remembered time of reinforcement to earlier time. In this study, acute metrifonate showed an increase in the peak time, suggesting a rightward shift, i.e. a change to the remembered time of reinforcement to a later time. This result was unexpected for a cholinesterase inhibitor. Metrifonate has been shown to improve cognitive performance in a variety of behavioural tasks (e.g. Schmidt *et al*, 1997). However the rightward shift suggests that cognitive processes were disturbed rather than that they were enhanced by the compound.

As hypothesised by Meck and Church (1984) changes in internal clock speed are transient whereas changes in the memory storage process are long lasting. A substance influencing internal clock speed should acutely shift the PT. The initial effect will disappear with repeated exposure as the animal learns to rescale time. On arrest of the drug, a rebound shift in the opposite direction should be observed. Contrarily, changes in the memory storage stage are only observed if the substance is chronically administered. The drug should permanently maintain the effects. A shift will slowly build up on drug administration, and slowly return to its original value when the drug is removed, no rebound shift will occur.

It has previously been suggested that cholinergic compounds affect the memory storage process which is thought to be linked to acetylcholine function in the frontal cortex. Therefore, we that a single dose of metrifonate would have no effect on the memory storage speed and any changes in timing would be produced by long term administration. Unexpectedly metrifonate affected timing behaviour in an opposite manner by producing an immediate shift to the right with a single administration and no effects with sub-chronic administration. It was suggested that effects of metrifonate may be observed after several days of administration but should only appear after a saline baseline (at least the time to build the representation of reinforced time in the reference memory). After a baseline has been established, several days of metrifonate administration should induce a modification of the temporal representation. However, in previous studies metrifonate has shown to produce a clear difference in behavioural performance in the Morris maze escape task on the first day of testing which indicates a fast onset of cognition enhancing effects (van der Staay, Hinz & Schmidt, 1996a).

Scopolamine, a muscarinic antagonist has been shown to impair behaviour in a variety of tasks (Buresova *et al*, 1986; Rush, 1988). In the peak interval procedure it has previously been shown to have an effect consistent with an increase in the remembered time of reinforcement, thus shifting the peak time to the right (Meck, 1983). In the present study, Scopolamine (1 mg/kg) produced a decrease in the spread which could possibly indicate a more accurate perception of the remembered time of reinforcement and an increase in the run rate which could suggest an increase in motivation for this timing task indicating a positive rather than a negative effect.

Cholinesterase Inhibition

A possible explanation could be that the 60 mg/kg dose of metrifonate produced first adverse side effects which caused the rightward shift as you would expect of a compound to impair the behaviour of the rat in this timing task. Previous studies with metrifonate have shown that the beneficial dose of metrifonate for improving cognition is between 10-30 mg/kg (Schmidt, 1997). It has been hypothesised that metrifonate may possesses a second mechanism of action that is unrelated to cholinesterase inhibition. In fact, it has already been shown that there is a discrepancy in the effective doses between behavioural and biochemical studies: cholinesterase inhibition by

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Effects of Strain or Sub-Chronic Metrifonate in the Two Choice Water Escape Task.

Abstract

The aim of the present study was to assess the performance of two strains of rat on the matching to position (MTP) and non-matching to position (NMPT) two choice water escape task. Also studied were the effects of the cholinesterase inhibitor (ChEI) metrifonate on the MTP task in order to evaluate the task as to its usefulness in identifying and characterising future putative cognition enhancers.

Both the Hooded Lister and the Harlan Wistar strain acquired the MTP task; with the Lister strain having a faster escape latency in the test run. However, there were no differences in choice accuracy. Neither strain could learn the NMTP version of the task.

Sub-chronic treatment with metrifonate (60 mg/kg) during MTP training tended to increase the escape latency during the test run, though large variances were observed within groups. No differences were observed in choice accuracy. To attempt to increase the difficulty of the task for the animals the retention interval was prolonged from 5 min to 2 hours. No effects due to a longer retention interval were observed. However, the longer in the retention interval combined with a change in platform position caused a transient performance deficit, with a decrease in escape latency in the control group.

No firm conclusions can be drawn from these results due the large variations within groups and the transient nature of the induced deficit.

Introduction

Animal studies that involve memory for trial dependent information to make correct choices are known as working memory tasks. However the subject normally uses both reference and working memory to solve these tasks. (Honig, 1978; Olton et al, 1979). The subject must use reference memory to retain the reinforcement contingency rules and/or general task requirements (win-stay, response alternation, matching to object etc.) that are in effect on all trials and use working memory to retain the trial dependent information (all previous choices of a given trial, the last response, sample stimulus etc.)

To determine effects on working memory rather than reference memory, one must train animals to a performance criterion and then test the subjects under conditions where the working memory requirements of the task are varied in difficulty. When a subject can meet performance criterion when working memory components are minimally difficult then the subject has acquired the reference memory components of the task. If the animal performs more poorly when the working memory components of the task are made more difficult while the reference memory components are held constant then the poor performance is probably due to the working memory components of the task (Means and Kennard, 1991). The working memory requirements of a task can be made more difficult by increasing the retention interval (Beatty et al, 1985; Ordy et al, 1988) or by increasing the amount of information to be retained (Aggleton et al, 1989)

Many of these working memory paradigms in rodents such as the radial arm maze (Olton and Samuelson, 1976), T- maze spontaneous alternation (Dember and Fowler, 1958, Gerlai, 1998), operant go/no-go alternation (Means et al, 1979) or operant delayed matching to sample (Dunnett et al, 1988) are appetitively controlled. T- maze win-shift or win-stay (Means et al, 1971; Stanton et al, 1984) is aversively motivated.

The water maze, a spatial reference memory task (Morris, 1981; 1984) in which rats tend to return on the second trial to the location in which an escape platform has

been found on the first trial has been adapted to test explicitly working memory (Buresova et al, 1985; Macutus and Murray, 1986). In this variation of the task (see Figure 1), the subjects are given trials that consist of two runs: an information run and a test run which are separated by a retention interval. The information run consists of a forced choice trial during which subjects are forced to the correct choice section by closing of the incorrect sections. The test run consists of a free choice trial, during which the doors to all choice sections are open. Incorrect first choices are punished by detaining the animal in the incorrect choice section for 20s or 30 s.

The task has many advantages of the Morris water maze, in that it involves no food or water deprivation which could interact with pharmacological or neural manipulations and ultimately alter behaviour that indirectly influence cognitive performance scores. Moreover, it appears to be less aversive than shock motivated tasks. The task provides a choice measure of performance as well as a latency measure. Choice is a better measure of cognitive processes than is response latency because choice is influenced less by variables such as age, or neurophysiological and pharmacological manipulations that alter motor ability and activity levels

The task has proven to be sensitive to age (Means and Kennard, 1991), oestrogen therapy in ovariectomised females (O'Neal et al, 1990) and gender (Means and Dent, 1991). There has also been observed improved performance in animals treated with Piracetam and BMY 21502, both putative cognition enhancers.

The present study is an examination of two different strains of rat on the matching to position (MTP) and non-matching to position (NMTP) two choice water escape task. It also to determines the effects of sub-chronic metrifonate on the matching to position water escape task. Finally, using metrifonate as a reference compound, the aim of this study is to evaluate whether the task is useful for assessing putative cognition enhancers as therapeutics of AD and other dementia type diseases.

9.1: Comparison of the Harlan Wistar and Hooded Lister strain of rat in the MTP and NMTP tasks in the two choice water escape maze

Procedure

Animals: 10 male Hsd Ola:LH rats and 10 Hsd Cpb:Wu (supplied by Harlan-Winkelmann, Borchen) were used. They were approximately 3 months old at the beginning of the experiment. Their weights ranged from 220-250 grams. The rats were housed in pairs in standard Makrolon™ cages in which food and water was continuously available, with a 12:12 hour light/ dark cycle, lights on at 07.00 am.

Apparatus and Methods: Cognitive testing took place in a circular white tub (material: polyethylene; inner dimensions: diameter 153 cm, depth 63 cm) that was filled with clear tap water at a temperature of approximately 22°C. A white polyethylene cylinder (diameter 17 cm), placed in the correct choice section, submerged 1.5 cm below the surface of the water served as an escape platform.

Each strain was divided into two groups of five animals of which one group received MTP training and the other group received NMTP training. After 22 sessions with the NMTP schedule, training in all animals was continued with the MTP schedule. All animals received approximately 47 training sessions.

All subjects were trained in one session per day. Each session consisted of two trials during which the escape platform was in the same choice section for MTP task. For the NMTP the escape platform was changed to the alternate choice section for the second run. Animals began all runs in the same start position that was located at the perimeter of the tank directly across from the centre of the top of the ‚T' divider (*see figure 1*). The first trial was labelled the Information run and the second, the Test run. For each run, an animal was released into the water facing the pool wall. Choices were forced during the information run i.e., the section not housing the escape platform was closed by a sliding panel. After climbing onto the escape platform and after staying there for a duration of 10 seconds, the animal was placed in a transfer cage during the retention interval, the time between the information

and the beginning of the test run. Choices were free for all subjects during Test runs; i.e., the sliding panel was positioned so that the subjects could enter either the left or right section. If a subject entered an incorrect choice section, the panel was closed and the animal was detained for 30 seconds before the panel was opened allowing it to swim to the correct choice section. Thus the rats were trained with a correction procedure in which incorrect choices were punished by 30 seconds of forced swimming in the correct section of the maze).

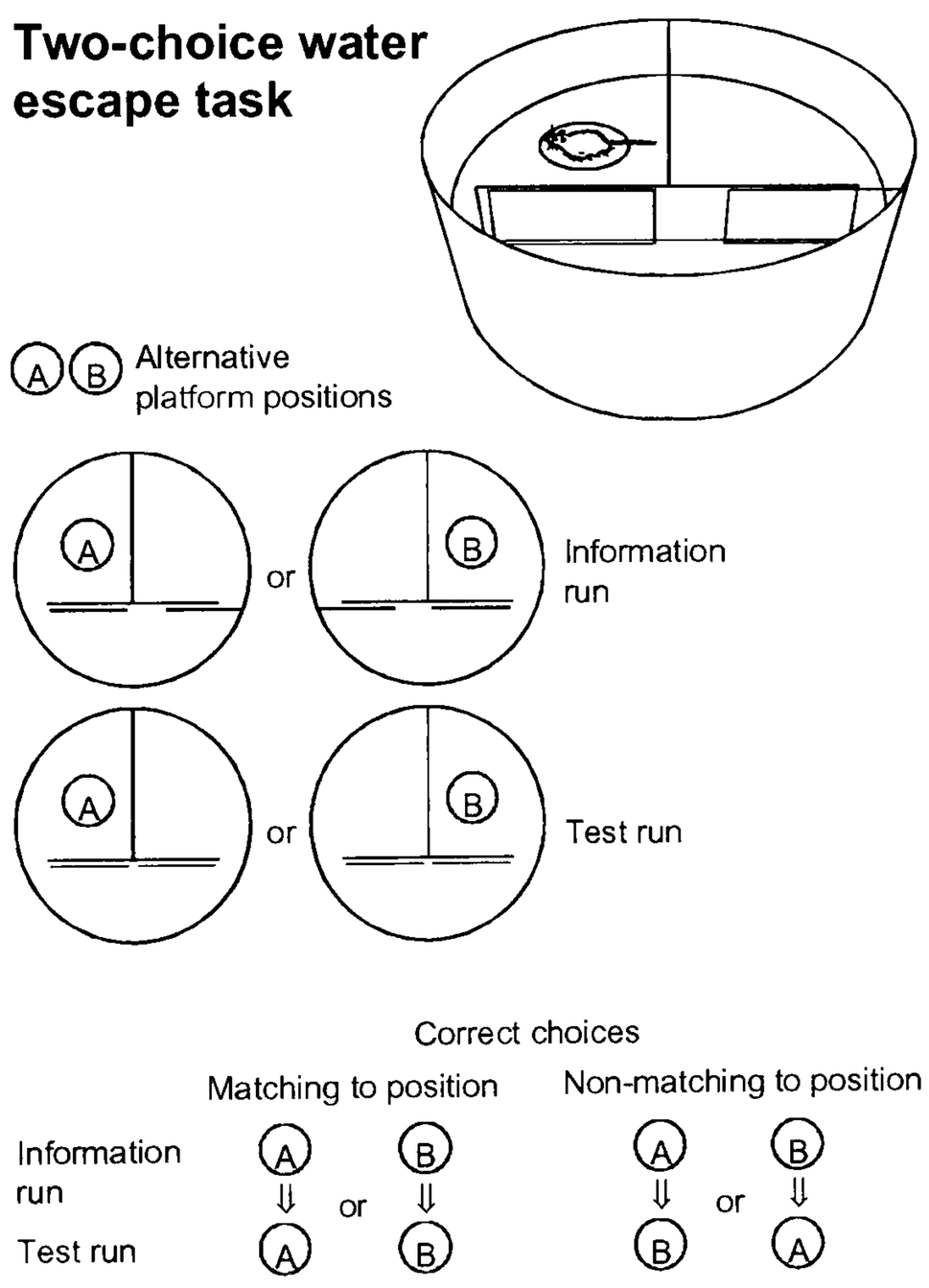

Figure 1. The two choice water escape task.

An animal was never retained in the incorrect choice section more than once in any given trial. A rat was removed from the water after 30 s to avoid reinforcing the incorrect choice. If an animal failed to reach the platform within 120 seconds, it was placed on the platform for 10 seconds by the experimentor. Throughout acquisition the retention interval was approximately 5 minutes.

Escape latencies (the time of release until the animals reached the platform) were recorded and first choice (whole body excluding tail in choice section) was recorded on both the information and test run.

Analysis: The latency to escape to the platform in all animals was assessed with a STRAIN*SESSION analysis of variance (ANOVA) with repeated measures over the first 10 Daily SESSIONS, supplemented by Duncan's multiple range test post hoc comparisons. A difference between groups was considered significant if the p value was below 0.05. In addition, the number of trials required to reach criterion (9 out of 10 sessions in succession without errors), the number of errors to criterion and the number of errors in the first 18 trials was analysed by ANOVA, with factor Strain, complemented by Duncan's multiple range post hoc comparisons. Also the correct choice escape latencies in the information run and test run of the animals that reached criterion was analysed by ANOVA with factor Strain, complemented by Duncan's multiple range post hoc comparisons.

Results

Experiment 1: Comparison of the MTP and NMPT of the Hooded Lister and Harlan Wistar strain of rat in the two choice water escape maze.

Matching to position: acquisition over the first 10 sessions

Escape Latency (see figure 2)

Information run: At the start of training the rats showed a similar level of performance (FIRST SESSION: $F_{1,8} = 0.15$, n.s). The average escape latencies of the two strains were similar across the training sessions (GENERAL MEAN: $F_{1,8} = 1.16$, n.s). Over the 10 sessions, both strains decreased their escape latency (SESSIONS: $F_{9,72} = 7.88$, $p<0.001$). The learning curves were not influenced by strain (SESSIONS by STRAIN: $F_{9,72} = 1.02$, n.s)

Test Run: The time to escape onto the platform were all similar at the start of training (FIRST SESSION: $F_{1,8} = 0.11$, n.s). The two strains of rat had a different mean escape latency during the first 10 training sessions (GENERAL MEAN: $F_{1,8} = 6.85$. $p<0.05$), the hooded Lister rats being faster than the Harlan Wistar rats. The strains decreased the escape latency during the test runs (SESSIONS:$F_{9,72}= 2.11$,

p<0.05). However the speed of learning was not different for the strain (SESSIONS by TREATMENT: $F_{9,72}$ = 0.76, n.s).

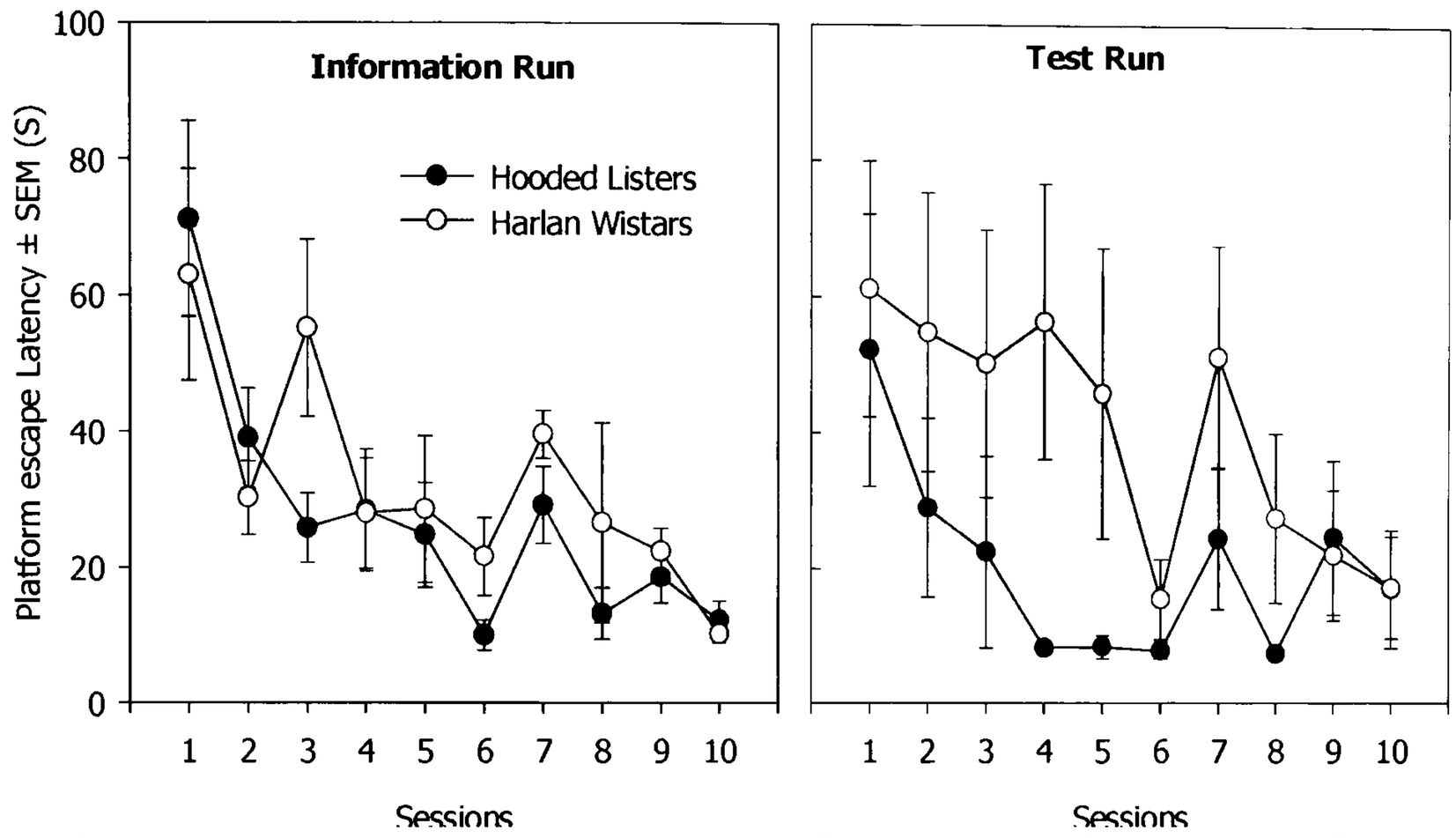

Figure 2: A comparison of Hooded Lister rats (N =5) and Harlan Wistar rats (N =5) on their performance in the two choice water escape task during the first 10 sessions of training on the MTP schedule. Session means and standard errors of the means (SEM) are depicted for latencies (s) to escape onto a platform in the information run (*left panel*) and in the test run (*right panel*).

Escape latencies for the correct choice trials (Figure 3): Averaged over the nine correct choice trials the escape latency in the information run was lower for the Harlan Wistar's than the Hooded Listers (t-test: t_8 =23.76, p<0.001). However, in the test run there was no difference in escape latency between the two strains (t_8 =1.36, n.s).

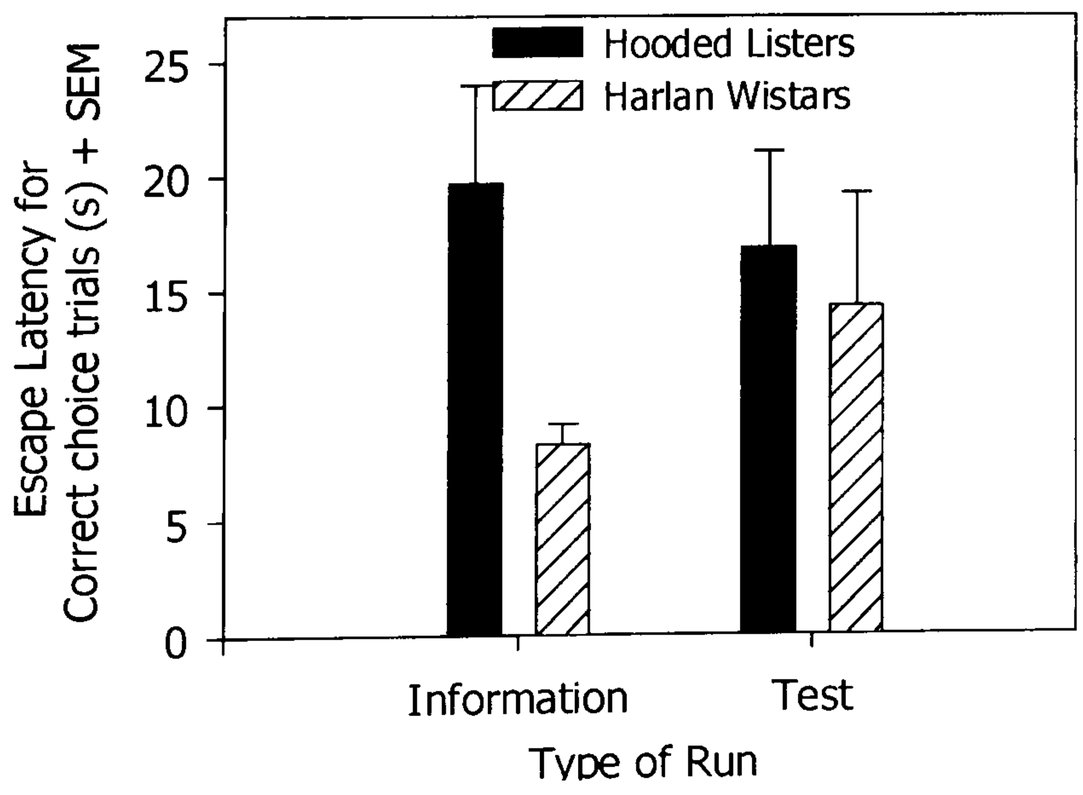

Figure 3: A comparison of Hooded Lister rats (N=5) and Harlan Wistar rats (N=5) on the performance in the two choice water escape task. The mean escape latencies for the nine correct choice trials + SEM in both the information and test runs.

Trials and Errors to Criterion: See figure 4. The two strains required almost identical number of trials needed to acquire the criterion of nine correct choices out of ten trials ($F_{1,8}$ = 0.00, n.s). The number of errors to criterion ($F_{1,9}$ = 0.02, n.s) and the number of errors in the first 18 trials ($F_{1,8}$ = 0.06, n.s) was also very similar between the two strains.

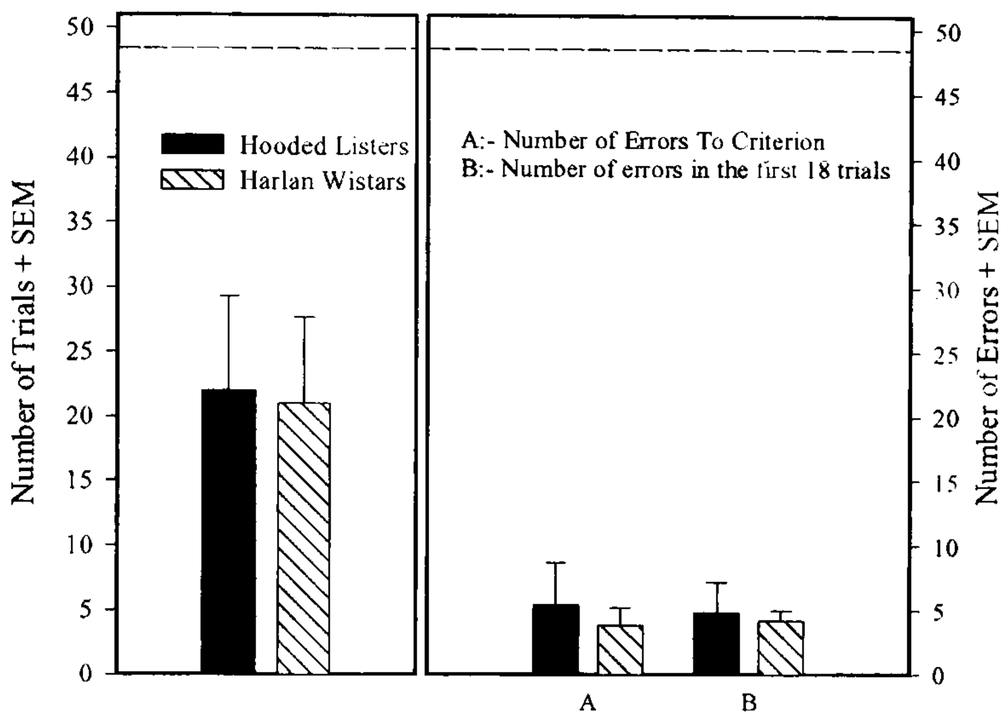

Figure 4: A comparison of Hooded Lister rats (N =5) and Harlan Wistar rats (N =5) on the performance in the two choice water escape task. The mean number of trials required + SEM (*left panel*). The mean number of errors to reach criterion +SEM and the number of errors in the first 18 trials (*right panel*).

Non Matching to position

*Escape Latency (*see figure 5)

Information run: At the start of training the rats showed a similar level of performance (FIRST SESSION: $F_{1,8}$ = 1.36, n.s). The escape latencies of the two strains were, on average similar during the course of training (GENERAL MEAN: $F_{1,8}$ = 1.71, n.s). In the course of training, both strains reduce their escape latencies (SESSIONS: $F_{9,72}$ = 13.43, $p<0.001$). This was not influenced by strain (SESSIONS by STRAIN: $F_{9,72}$ = 1.78, n.s)

Test Run: The time to escape onto the platform were all similar at the start of training (FIRST SESSION: $F_{1,8}$ = 0.11, n.s). The escape latencies of the two strains were, on average similar during the course of training (GENERAL MEAN: $F_{1,8}$= 6.85,$p<0.05$).

Over 10 sessions the escape latency during the test run did decrease only slightly (SESSIONS: $F_{9,72}$= 1.76, $p<0.05$) and this decrease was similar for both strains (SESSIONS by STRAIN: $F_{9,72}$ 1.69, n.s).

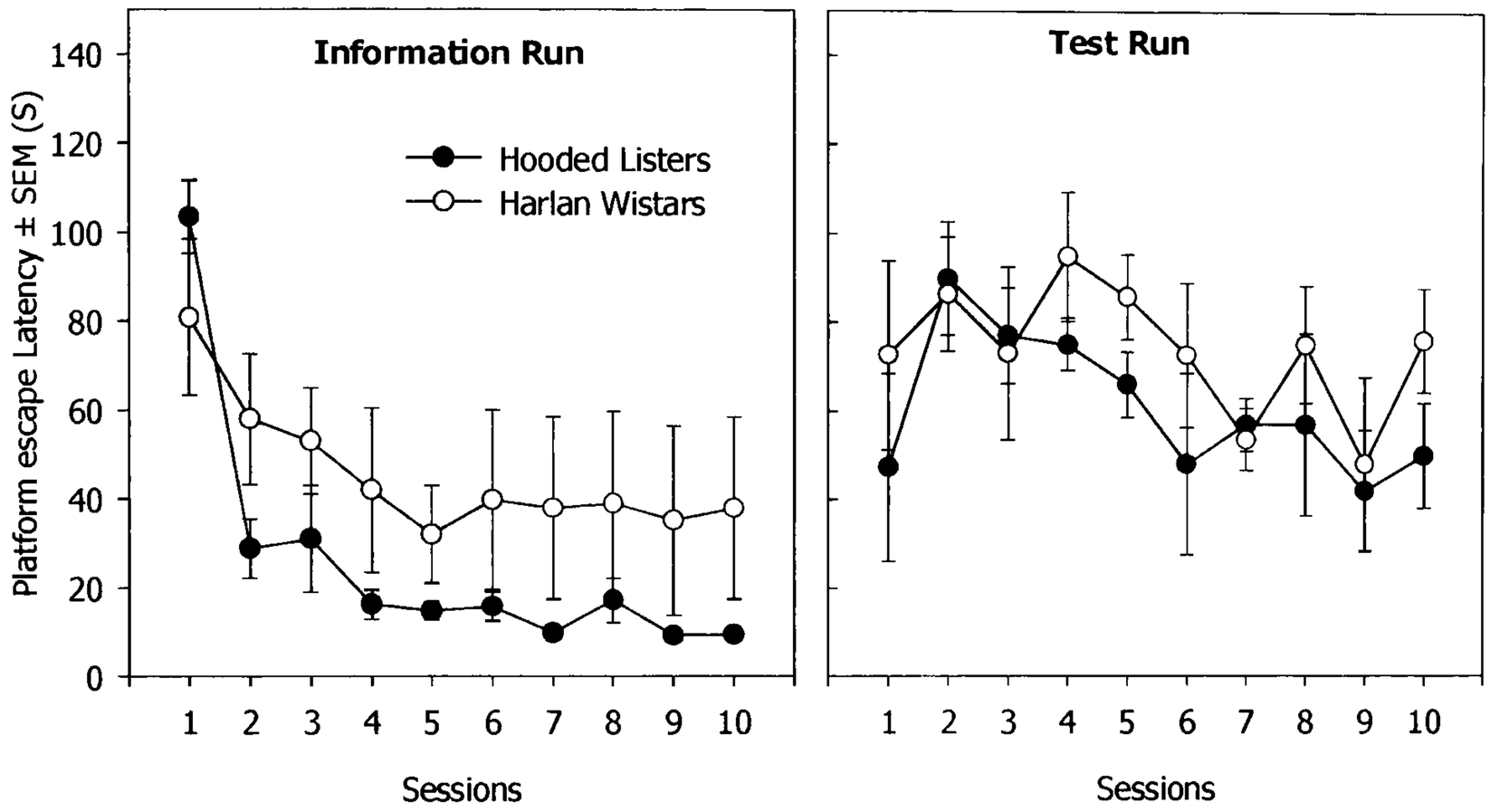

Figure 5: A comparison of Hooded Lister rats (N =5) and Harlan Wistar rats (N =5) on the performance in the two choice water escape task during the first 10 sessions of training of NMTP. Session means and standard errors of the means (SEM) are depicted for latencies (s) to escape onto a platform in the information run (*left panel*) and in the test run (*right panel*).

Trials and Errors to Criterion: Neither the Hooded Lister nor the Harlan Wistar strains reached a criterion of nine correct choices out of ten sessions in the non-matching to position two choice water escape task within 21 training sessions *(Data not shown*). This was the reason for discontinuing training on the NMTP schedule.

9.2: The effects of Sub- Chronic metrifonate treatment in the hooded Lister rat in MTP in the two choice water escape task

Animals: 35 male HsdOla:LH rats (supplied by Harlan- Winkelmann, Borchen) were used. They were approximately 10 weeks old at the beginning of the experiment. Their weights ranged from 220-250 grams. The rats were housed in groups of two in standard Makrolon™ cages in which food and water was continuously available, with a 12:12 hour light/ dark cycle, lights on at 07.00 am.

Drug Administration: Rats were treated orally once daily with 30 (N=9), 60 (N=7) or 100 (N=9) mg/kg Metrifonate in an application volume of 5 ml/kg from day one of training. Controls (N= 10) received the vehicle Sodium citrate buffer, pH 5.5 under identical conditions. Metrifonate was administered 30 minutes prior to the start of the session.

Apparatus: Apparatus and methods are as described in *experiment 1* with the exception than only MTP was studied and that the retention interval was prolonged from 5 minutes to 2 hours after the animals had reached criterion

Analysis: The latency to escape to the platform latencies in the information run and test run was assessed with a TREATMENT*SESSION analysis of variance (ANOVA) with repeated measures over 5 Daily SESSIONS, supplemented by Fischer's Least Significant Difference (LSD) post hoc comparisons. A difference between groups was considered significant if the p value was below 0.05. The number of trials required to reach criterion (7 out of 8 training sessions without errors) and the number of errors to criterion was analysed by ANOVA, with factor treatment complemented by Fischer's LSD post hoc comparisons. The correct choice escape latencies in the information run and test run of the animals that reached criterion was analysed by ANOVA, with factor treatment complemented by Fischer's LSD post hoc comparisons. Also to determine the effects of prolonging the retention interval, the escape latencies of the test run was analysed by ANOVA with factor treatment with repeated measures over sessions. Session 1 was the final day with a 5 min retention interval. In session 2 is the day at which the retention interval was prolonged to 2 hours. In session three, the retention interval remains at 2 hours and the platform position is changed from left to right and session 4 and 5 is with a 2 hour retention interval and the platform position on the right side of the maze.

Results

Escape Latency

Information run: At the start of training the groups of rats showed a different level of performance ($F_{3,31}$ = 10.46, $p<0.05$). Averaged over the first 5 sessions,

metrifonate influenced on the escape latency (GENERAL MEAN: $F_{3,31}$ = 2.62, $p<0.05$). Post hoc analysis by Fischer's LSD revealed that 60 mg/kg Metrifonate had, on average, a shorter platform escape latency than the vehicle treated group. The escape latencies of metrifonate treated groups generally decreased during the course of training (SESSIONS: $F_{4,124}$ = 11.31, $p<0.001$) and this decrease was influenced by metrifonate treatment (SESSIONS by TREATMENT: $F_{12,124}$ = 4.16, $p<0.001$).

Test Run: The time to escape onto the platform were slightly different at the start of training (First Session: $F_{3,31}$ = 3.66, $p<0.05$). The average escape latencies were not affected by metrifonate treatment (GENERAL MEAN: $F_{3,31}$ = 0.30, n.s). Post hoc analysis by Fischer's LSD revealed that 60 mg/kg Metrifonate had a shorter platform escape latency than compared to the vehicle treated animals. Across the 5 sessions the escape latencies became shorter (SESSIONS: $F_{4,124}$= 7.65, $p<0.001$). The decrease of escape latencies was affected by metrifonate treatment (SESSIONS by TREATMENT: $F_{12,124}$= 3.18,$p<0.001$).

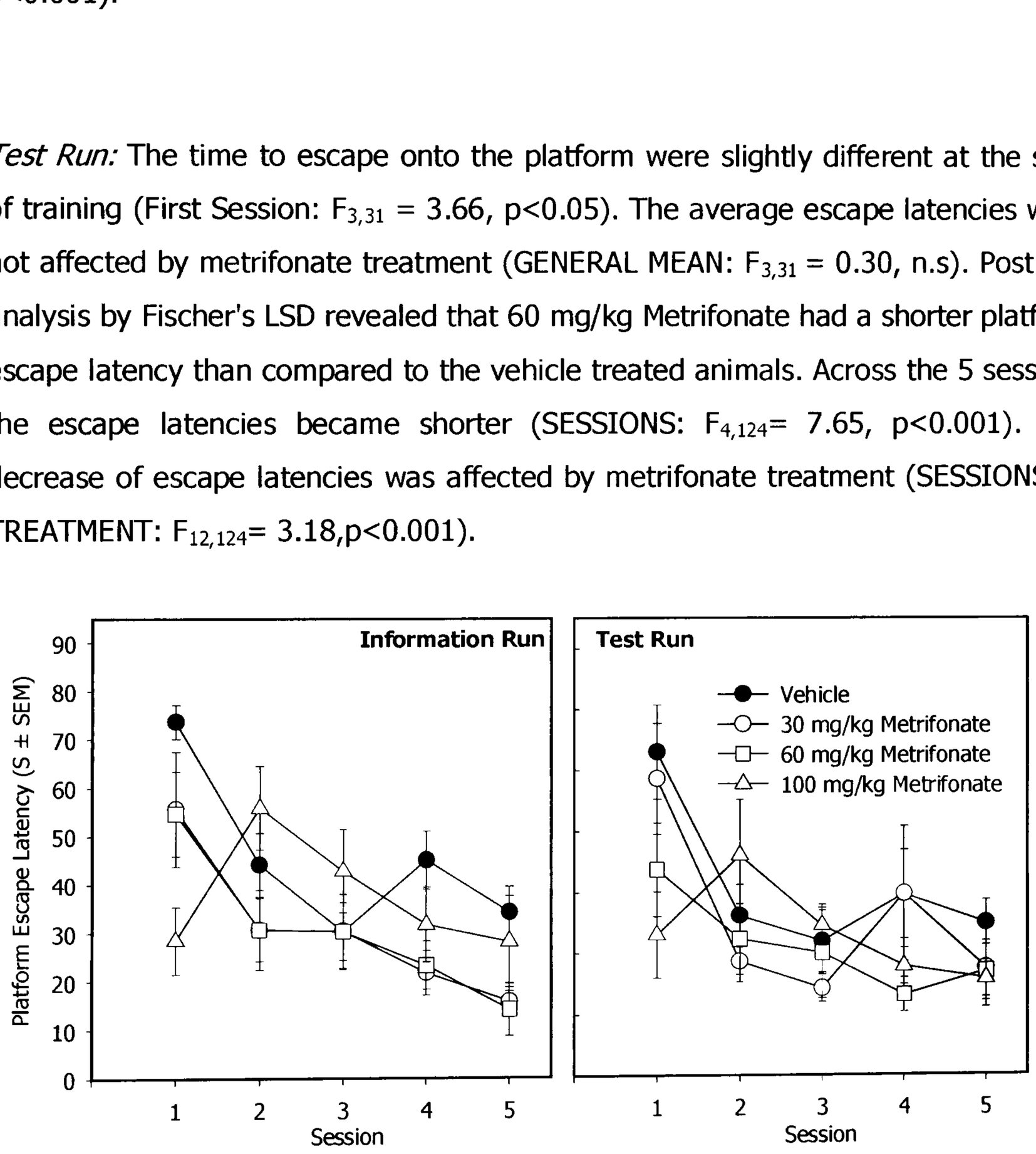

Figure 6: The effects of metrifonate (30, 60 and 100 mg/kg) in Hooded Lister rats on the performance in the two choice water escape task during the first 5 sessions of training of MTP. Session means and standard errors of the means (SEM) are depicted for latencies (s) to escape onto a platform in the information run (*left panel*) and in the test run (*right panel*).

Escape latencies for the correct choice trials: (*See figure 7).*

Averaged over the seven correct choice trials of animals that had reached criterion, the escape latency in the information run was not affected by metrifonate ($F_{3,31}$ =0.62, n.s). However, in the test run there were differences in escape latency between the metrifonate treated groups ($F_{3,31}$ = 3.53, $p<0.05$). Post hoc analysis by Fischer's LSD revealed that the 30 and 60 mg/kg treated group had a longer escape latency than the vehicle treated group.

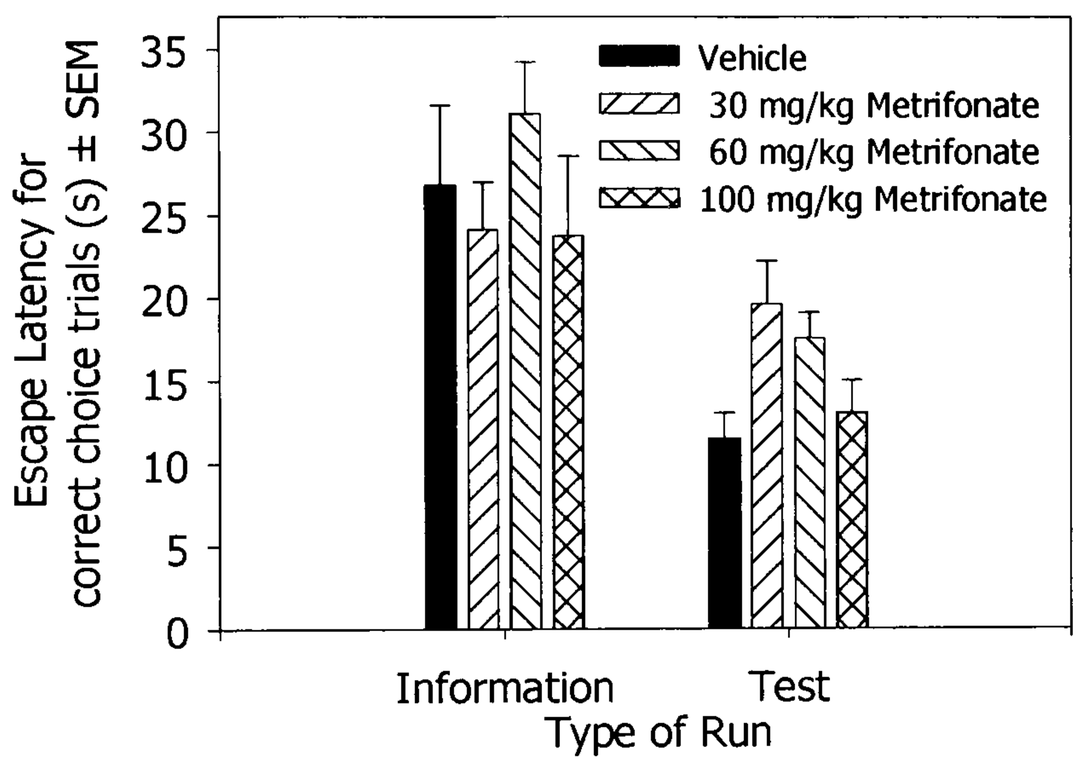

Figure 7: The effects of Metrifonate (30, 60 and 100 mg/kg) on the performance in the two choice water escape task. The mean escape latencies for the correct choice trials ± SEM in both the information and test runs.

Trials and Errors to Criterion: All groups required almost identical number of trials needed to acquire the criterion of seven correct choices out of eight trials ($F_{3,31}$ = 0.40, n.s). The number of errors to the criterion ($F_{3,31}$= 0.27, n.s) was also very similar between metrifonate treated groups (*Data not shown*).

Retention Interval

Test Run: (See figure 8). On the final day of training with a retention interval of 5 min (Session 1) there was a similar escape latency in the test run between all groups ($F_{3,31}$ =1.11, n.s) and this was also observed on the first day (Session 2) of a retention interval of 2 hours. ($F_{3,31}$ =1.11, n.s). However the escape latencies developed differently between groups (SESSIONS: ($F_{4,12}$ =4.97, $p<0.05$) and this was affected by metrifonate treatment (SESSION by TREATMENT ($F_{4,112}$ =2.83,

$p<0.05$). Changing the platform position from left to right in the third session, in addition to prolonging the retention interval, affected the performance ($F_{3,31}$ $=3.43, p<0.05$). Post hoc analysis revealed that the vehicle control group had a longer escape latency than the three metrifonate treated groups, which did not differ from one another.

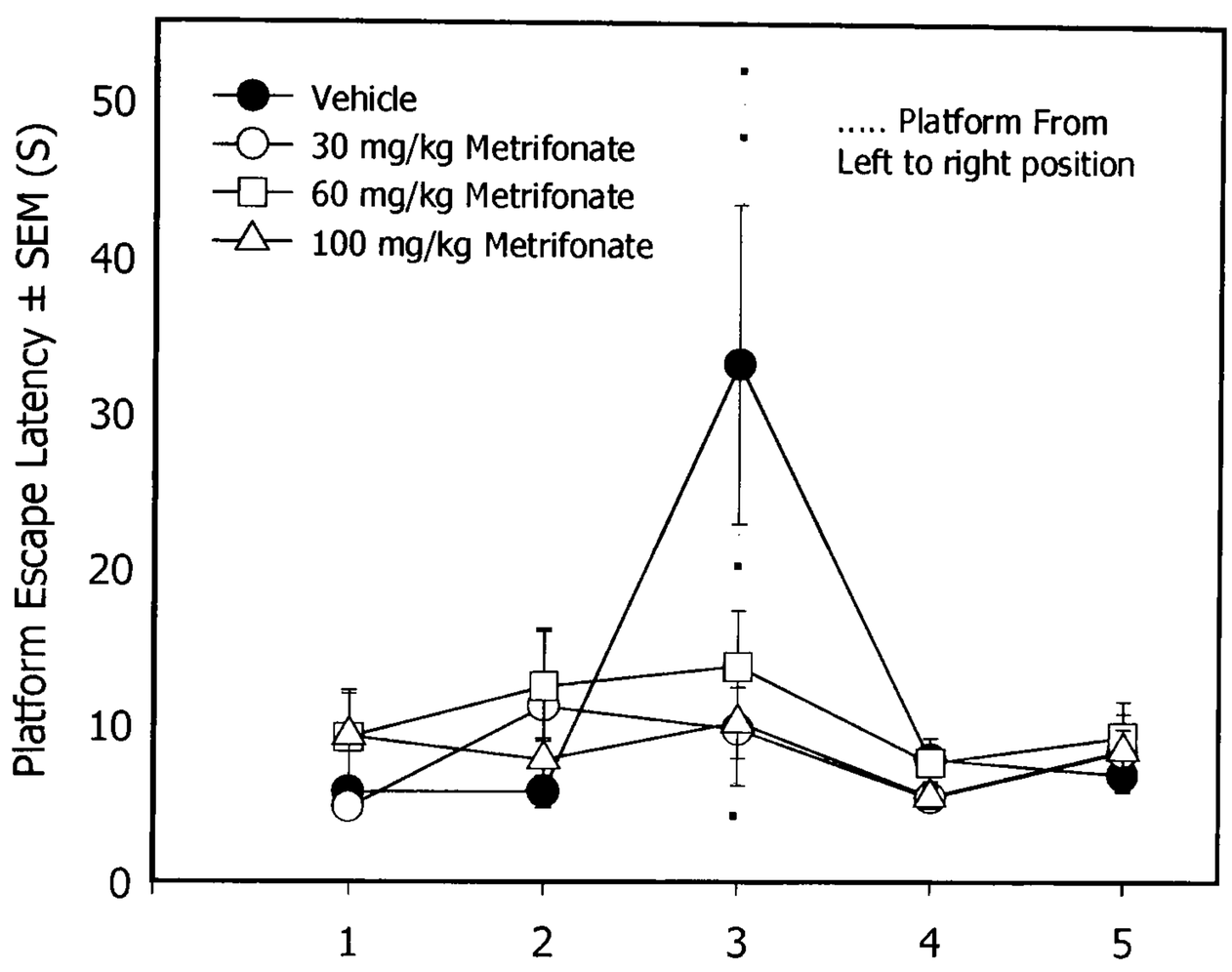

Figure 8: Effects of metrifonate (30, 60 and 100 mg/kg) on the test run platform escape latency. Session 1: the final retention Interval of 5 minutes. Session 2: prolonging the retention interval to 2 hours. Session 3: Changing the platform position from left to right with a retention interval of 2 hour. Sessions 4 and 5: the platform position staying in the right position with a 2 hour retention interval. Results are ± SEM, N = 7-10.

Discussion

The aim of the present study was to compare the efficiency of two strains of rat in acquiring either the matching to position (MTP) and non matching to position (NMTP) two-choice water escape task and to determine the effects of metrifonate, a cholinesterase inhibitor in one of these strains on the matching to position water escape task.

Strain Comparison

The results show that both the hooded Lister's and the Harlan Wistar strain of rat acquired the working memory matching to position (MTP), and that the hooded

Listers had a faster escape latency in the test run. They did not; however make fewer mistakes to the choice section compared to the Harlan Wistar's suggesting that both strains had a similar ability to retain the correct escape choice over the 5 min retention interval.

Neither strain could acquire the non matching to position (NMPT) task and it was observed that both strains were faster to the platform in the test run and information run in MTP compared to NMTP (data not shown). It was also observed that when the animals changed over to the MTP from NMTP task after 21 sessions there was no further improvement in learning the MTP task and that they performed worse than animals that had been trained on the MTP directly (Data not shown).

Sub chronic metrifonate administration.

In this study the group of rats treated sub-chronically with metrifonate (60 mg/kg) had a shorter escape latency than the vehicle treated group in the information run. However, no effects of metrifonate were observed in the test run. Also, as with the strain comparison, there was no difference for the number of trials or the number of errors to criterion.

All animals performed with 80-100 percent accuracy during criterion trials that involved a 5-minute retention interval (RI). This accuracy remained with the introduction of a retention interval of 2 hours (Data not shown). There were also no changes in escape latencies between treatment groups. However when the platform position was changed from left to right before the information run of a session, the percent accuracy of the vehicle treated animals was reduced to 30%. In conjunction to this there was a large increase in the escape latency. The metrifonate treated groups showed no changes in either the percent accuracy or escape latency. This suggests a transient impairment in the vehicle group when two parameters are changed: RI and platform position. When only one of these two procedural changes was introduced, no impairments were observed.

A final observation in both experiments was that though the Hooded Listers were faster to escape onto the platform in all test trials, the escape latency was lower for the Harlan Wistar's when only the correct choice trials was considered. This was

also observed with the groups treated with 30 and 60 mg/kg metrifonate. Perhaps the animals take more time to make the correct choice.

Discussion of task

Collectively, both experiments showed that the most natural response of the rats given pairs of trials is to follow the same path for every trial. The animals appear to be using a strategy in which the immediately preceding trial is used to the extent that it influences the collective memory of all proceeding trials. They also adopt a course that takes them to the escape platform in a relatively short time. The first experiment showed that the animals could not learn a non-matching position strategy in the water maze.

The delayed matching procedures have several advantages over other paradigms frequently used to study mnenomic processes in animals, such as shock motivated avoidance procedures. They can allow selection of a clearly defined element of behaviour that can be studied by specific task variables. In this two choice water escape task three different aspects of behaviour can be studied.

Escape latency can be used to determine changes in reference memory. The subject must use reference memory to retain the reinforcement contingency rule (win-stay, response alternation, matching to sample etc.) that is in effect on all trials. Another dependent variable of interest is response accuracy. Changes in response accuracy are thought to reflect changes in non-mnenomic processes such as motivation, attention and /or motor processes (van Hess, et al, 1996)

Finally, to determine effects on working memory rather than reference memory, one must train animals to a performance criterion and then test the subjects under conditions where the working memory requirements of the task are varied in difficulty. The working memory requirements of a task can be made more difficult by increasing the retention interval (Beatty et al, 1985; Ordy et al, 1988).

These variables can allow dissociation between mnemonic effects and other factors that may alter performance. However, in this study changes were only observed in

reference memory (escape latency) with no changes in choice accuracy (attention) and only a transient change in working memory in the vehicle treated group when 2 parameters are changed together i.e. RI and platform position. Also the task has some disadvantages in that training the animals to stable baseline performance is time consuming and ceiling effects are critical for interpretation of data, particularly where the animals escape latency is already fast and accuracy is at a high level. The effects of metrifonate did not show any cognition enhancing properties, which have been observed in previous behavioural experiments (van der Staay et al, 1996). From this study we are unable to evaluate the effectiveness of this task for assessing future cognition enhancers. Further study is required, for example by introducing changing RI earlier during training or possible by inducing pharmacological deficits such as those due to scopolamine.

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SUMMARY AND CONCLUSIONS

Introduction

Animal tests of cognitive function have the potential to assist in drug discovery both directly as screening procedures, and indirectly through their fundamental role in the investigation of the neurochemical basis of cognition.

The cholinergic system is thought to be the primary system involved in learning and memory. Alzheimer's disease (AD) is a chronic neurodegenerative disorder characterised by a progressive decline in cognitive function, including loss of memory and changes in behaviour. The causes of the disease are as yet unknown. However, central cholinergic hypofunction is thought to be a major component of the disease (Bartus *et al,* 1982). Several lines of evidence support this notion that a loss of cholinergic function contributes to the cognitive deficits seen in AD (Bierer, *et al,* 1995). The most successful of these strategies at this time is to increase the amount of acetylcholine by reducing its degradation by cholinesterase inhibitors (ChEI's).

Metrifonate is a second generation of cholinesterase inhibitors currently available (See chapter 1). Firstly, we looked at further biochemistry of metrifonate to determine how the compound develops tolerance and whether this is a central or peripheral phenomenon. The aim of this study to confirm the behavioural effects of metrifonate, serving as a reference cognition enhancer in known behavioural models in the rat and second to determine these effects in another species i.e. the mouse. The third aim was the assessment of new methods which allow to test and characterise future cognition enhancers using the reference compounds.

Summary of Results

In chapters 2 and 3 the biochemical activity of metrifonate was further characterised, specifically in relation to the development of tolerance after repeated exposure to the compound. With administration of central or peripherally acting cholinergic compounds to animals pre-treated with metrifonate it was found that the development of tolerance is a centrally acting phenomenon and specifically related to the cholinesterase molecule. Further study on the gene expression of acetylcholinesterase and butyrylcholinesterase showed that there is a two fold up

regulation of the gene increasing the amount the these two proteins. However, there is reported to be no changes in acquisition in retention of behavioural tasks. A second mechanism of action for metrifonate was suggested and recent reports have revealed a high selectivity of metrifonate for the substance acylpeptide hydrolase (ACPH, Richards *et al*, 2000; Richards *et al*, 1999). The *in vitro* sensitivity of DDVP (Metrifonate active metabolite) for ACPH is about 7 times greater than for AChE, a higher sensitivity has also been confirmed *in vivo*. When 47% inhibition of AChE is obtained by DDVP treatment, ACPH is nearly totally inhibited i.e. to 93% (Richards *et al*, 2000). ACPH is a peptidase and not an esterase like AChE, which broaden the range of mechanism of organophosphorous ChEI.

The enzyme catalyses the hydrolysis of N-acetylated amino acids from short peptides to form an acylamino acid and a peptide with a free NH_2-terminus. Preference for N-acetyl-methionyl-, alanyl-, glycyl-, and seryl-containing peptides (common N-terminal residues for cystosolic proteins) has led to the suggestion that the enzyme is important in protein catabolism or removal of N-acylated amino acids from nascent polypeptide chain emerging from the ribosome (Raphel *et al*, 1999).

The role of ACPH in neuropeptide turnover had been put forward as a new and plausible mechanism for the cognitive-enhancing effects of certain organophosphorous compounds (Ray *et al*, 1998). However, further research in this direction was inconclusive (unpublished results).

In chapter 4, the effects of the cholinesterase inhibitor metrifonate was observed after either chronic or acute administration in the passive avoidance task which is commonly used as a fast screening tool. Acute metrifonate had no effect on the performance deficits in the retention session. The chronic study (experiment 4.2.) demonstrated that the reference compound effectively inhibited the scopolamine-induced deficit. Although the cholinesterase inhibitor was effective in antagonising the scopolamine induced deficits, it was concluded that a single test cannot be sufficient to determine the full cognitive enhancing properties of the reference compounds. Therefore, further verification, using more sophisticated tests was required.

Chapter 5 studied the effects of sub-chronic metrifonate in neurologically normal rats in the standard Morris water escape task. The compound improved spatial memory. Previous studies with metrifonate have shown cognitive improvement in various animal models (Blokland *et al*, 1995; van der Staay *et al*, 1996; Kronforst *et al* 1997) after subchronic administration. One explanation for the negative findings was that the performance of the rats in this study may have imposed a ceiling effect (i.e. the rats had reached maximum performance levels) any and therefore changes in behaviour was masked. Administration of acute metrifonate in neurologically normal mice also did not improve spatial reference memory, however sub chronic administration (30 mg/kg) showed beneficial effects: metrifonate treated mice escaped to the platform faster than the vehicle treated control group.

An important point of this chapter is that compounds that improve memory may not necessarily improve performance in neurologically normal animals and therefore to further assess the effects of cholinesterase inhibitors was considered necessary to use animals that have a cholinergic hypofunction.

In chapter 6, we investigated the effects of acute treatment of metrifonate on water maze navigation in mice with scopolamine-induced amnesia. Metrifonate had no ameliorating effects on the deficit induced by scopolamine. The unsuccessful use of metrifonate in ameliorating a scopolamine induced deficit possibily indicated that pharmacokinetic interactions between scopolamine and future, unknown cognition enhancers may interfere with behavioural observations. It was suggested that the alternative is excitotoxic lesions, which can be aimed to more specific regions of the brain involved in learning and memory rather than the general effect of scopolamine

In chapter 7 metrifonate was assessed on spatial performance of rats with bilateral lesions of the entorhinal cortex (EC). Treatment with metrifonate (30 mg/kg) in rats with bilateral EC lesions improved performance in the spatial discrimination task compared with that of the sham lesioned control group. The present experiments indicate that under conditions of pathological impairment of brain structures such as entorhinal cortex lesion, future ChE-Is might produce beneficial effects on learning and memory.

In chapter 8, the Peak interval procedure was used to assess the effects of the ChEI's on time estimation. A single administration Metrifonate (60 mg/kg), shifted the peak time to the right, indicating an impairment in time estimation. This could have been due to unobservable adverse side effects. Sub-chronic administration of metrifonate had no effect on time estimation.

In chapter 9, we investigated the differences between two strains of rat and the effects of sub chronic metrifonate treatment in the two choice water escape task. Both the Hooded Lister and the Harlan Wistar rats acquired the matching to position task, with the Lister strain being faster in escaping onto the platform. However there was no difference in choice accuracy and neither strain could learn the non-matching to position version of the task. Sub chronic metrifonate treatment (60 mg/kg) increased the escape latency to reach the platform. However there was no difference in choice accuracy. Extending the retention interval from 5 min to 2 hours had no effects. No specific conclusion could be drawn as to the effectiveness of this task in assessing future cognition enhancers. Further study is required with the inclusion of deficits earlier in the training and/or the inducement of scopolamine or lesion deficits.

Evaluation of the tasks used

When using animals to model the behavioural impairments of humans, the choice of task and manipulation are of considerable importance. The tasks discussed below cover relevant aspects of information processing. Their value for characterising cognitive impairments and evaluating putative cognition enhancers is discussed below.

Passive Avoidance task

The passive avoidance task is considered to be a fast screening tool (Iversen, 1997). However, as discussed in chapter 2, the validity of this task is extremely low. Though mnemonic processes are affected in this task, there are also effects on stress, anxiety, emotionality, changes in motivation, and in arousal levels etc (van Dijken, 1992). Compounds, which give positive results in this task, do not necessarily improve cognition in other, more complex tasks.

With the passive avoidance task, changes in the latency to enter the dark compartment in the retention session are considered to be a measure of retention performance. Long latencies are interpreted as reflecting good retention performance and short latencies are indicative of poor retention or possibly amnesia. However, in experiment 2.1 in chapter 2, metrifonate had no effect on the latency to enter the dark compartment, though it did increase the amount of time spent in the light compartment. As previous studies have shown positive effects with metrifonate (Riekkinen *et al* 1996; Itoh, *et al*, 1997), it might be useful to look at the time spent in the light compartment during the retention session as an additional index of retention. For example, the animals might have entered the dark compartment fast due to increased arousal (the apparatus has been associated with the aversive event of a foot shock). Then, however, the animal immediately escaped from the dark to stay mostly in the light. I.e. there might have been recognition of the dark compartment, followed by escape, and avoidance of the compartment.

Morris water escape task

Spatial discrimination tasks in mazes, most commonly the Morris water maze are among the most frequently used tests to detect potential cognition enhancers and to assess their potency (Merlini *et al*, 1989; Andrews, 1996). An advantage of the Morris water escape task is that no food deprivation is necessary for motivating the animals to solve the task. However, other aspects of the task have received criticism. One of the main disadvantages of the test (Block, 1999; Stewart and Morris, 1993 and Wenk, 1998), is that it requires the tested animals to flight from an aversive environment seemingly devoid of escape routes. The immersion of the animals into the water and the initial sensation of being trapped in it may cause considerable stress during the first stages of the test. Although Stewart and Morris (1993) have argued that stress would be minimal at reasonable water temperatures. To reduce this kind of interference, the use of a short adaptation procedure to accustom the animals to immersion in water has been recommended.

The escape latency is the most used parameter for assessing water maze behaviour. However, this measure is dependent on the speed in which the animal negotiates the maze. Other aspects need to be measured to determine the affects of possible cognition enhancers. For example if the swimming speed has been affected by an

experimental manipulation, the distance swum before escaping onto the platform is a more useful measure of spatial navigation performance. Looking at search patterns/strategies of the animals would provide additional relevant information about the effects of compounds on spatial navigation performance (Whishaw, 1985; Lipp and Wolfer, 2000).

This was observed in chapter 6 where scopolamine induced deficits caused thigmotaxis, or wall hugging behaviour. The chance of finding the platform in the water tank decreases with increasing thigmotaxic behaviour, because the animal does not negotiate the maze (Paylor et al, 1990; Whishaw et al, 1987; Cain and Saucier ,1996).

The MWM test is one of the most frequently used research tools The test has a number of advantages, and has been used in such an impressive variety of applications that it might also rank as one of the best assays for spatial learning and memory in laboratory rodents. its disadvantages and limitations, however should be recognized as well, and researchers should continue to look for new and better alternatives

Timing behaviour

The timing discrimination performance in the Skinnerbox was suggested to be a tool to test cognition enhancing properties of new therapeutics. The timing behaviour can be assessed by a peak interval procedure (see chapter 8). Rats are trained to respond to a discrete fixed interval (FI) procedure in which a lever is presented and the animal is free to respond at any time, but only the first response after a fixed duration (e.g. 20 seconds) is reinforced. In the peak interval (PI) procedure some trials are identical to the FI procedure where others consist of the lever presented for a time that goes beyond the fixed duration (e.g. 50 seconds in our experiments, and typically at least twice plus a random duration of the fixed interval) and no reinforcement is given (Catania, 1970; Roberts, 1981; Church, *et al,* 1991).

The maximal response rate can shift to a longer or shorter interval as a consequence of experimental manipulations (e.g. choline, Meck and Church, 1987a; physostigmine, Meck and Church, 1987b; Scopolamine, Meck, 1983). Cognition enhancers are expected to shift the peak time to the left, reflecting a change in time estimation. However, our study could not conclude as to the effectiveness of this paradigm. Training is lengthy, thus as a screening tool, the throughput of testing compounds on the acquisition of the timing task is low. Also, data analysis is relatively complex and results are difficult to interpret.

The timing task could be used to further evaluate compounds rather than as an initial screening paradigm, it could be used in conjunction with other behavioural paradigms. It might help to further characterise compounds, which are supposed to improve cognition.

Two choice water escape task

The Two choice water escape task is an extension of the standard Morris water escape task in which the animals have to choose as to which section of the maze to enter in order to reach a platform (see chapter 9). The task itself has great potential in that it can measure three different aspects of performance. Reference memory can be measured by the escape latency that is in effect on all trials as this reflects that the animal has learned that it can swim to a platform to escape from the water. Changes in response accuracy are thought to reflect changes in non-mnemonic processes such as motivation, attention and /or motor processes but also in reference memory (van Hest, *et al*, 1996). Finally, the effects on working memory, by introducing increasing difficulties in the task, i.e. by increasing the retention interval.

However, performing this task I found that training the animals to stable baseline performance is time consuming. Only changes in reference memory were observed. This does not preclude that the task is not effective in assessing future cognitive enhancers; it does suggest further work is required. For example, the use of reference compounds other than metrifonate is required and the effects of introducing deficits by scopolamine or by selective brain lesions need also to be studied. Previous studies with bilateral lesions of the hippocampal formation

produced deficits, with the rats, taking longer and swimming farther to find the platform on the test swims (Glen and Mumby, 1998), i.e. the task appears to depend on normal hippocampal functioning,

Evaluation of the animal models used

Animal models remain the main tool to characterise compounds that can be used as therapeutics in the treatment of behavioural deficits caused by disease (Sarter *et al*, 1992; Allain, 1997). The focus of animal models of AD has been on identifying the areas of the brain and neurotransmitter systems thought to be involved with memory. The cholinergic system has received the most attention, because of studies showing degradation of the basal forebrain cholinergic system in AD (see chapter 1).

Normal animals as models of cognitive deficits and/or AD

Normal animals present no observable behavioural deficits, and cognitive effects of a compound may be masked due to the restricted range of potential of performance. i.e. a ceiling or floor effect in the test used. If no effects are observed in normal animals, the compound might still ameliorate cognitive impairments in patients (Decker, 1995). However, it is important to determine as to whether that particular strain or species has underlying, undetectable deficiencies. The most important point is that compounds which improve cognitive function in normal animals may be classed as cognitive enhancers. However, because there are no clinical symptoms of AD in these animals, the potential of a compound as disease modifiers remains unknown.

Animals with Scopolamine-induced performance deficits as a model for AD

The most widely used model of the memory impairment of AD is the administration of the muscarinic antagonist, scopolamine, which has been shown to interfere with learning and memory in humans (Beatty *et al.*, 1986) and experimental animals (Stevens, 1981; Sutherland, 1982). In this study, scopolamine was shown to induce deficits in the passive avoidance task in rats (chapter 4), and the Morris water escape task (chapter 5) in mice.

Although scopolamine appears to be an effective tool in producing deficits on tests of memory, it also acts on central and peripheral sites of autonomic and motor

control. In addition, the cholinergic system is probably involved in psychological processes other than memory that may affect performance on tasks that we infer as memory (Fibiger, 1991; Fibiger *et al*, 1991). Effects on acquisition might be reflection of disrupted attentional processes (Cheal, 1981) or information processing. The various actions of scopolamine must be considered when attempting to interpret its behavioural effects.

The effects of scopolamine on a variety of tasks are robust and reproducible. However, there are differences between the scopolamine model and the symptoms of Alzheimer's disease. The impairments are induced by post-synaptic blockade rather than by pre-synaptic destruction of cholinergic neurons, whereas in the case of AD this is largely a pre-synaptic degenerative phenomenon (Decker, 1995).

Animals with lesions as a model for AD

Degeneration of the basal forebrain cholinergic neurons occurs early in the course of AD and is correlated with cognitive deficits (Coyle *et al.*, 1983; Winkler *et al.*, 1998). Cholinergic lesion paradigms have been used to study the role of the cholinergic system in cognitive function (Nabeshima, 1993; McDonald ant Overmier, 1998). Many types of acute manipulations, including electrocoagulation, use of excitotoxins, transection of the fimbria fornix and treatment with cholinotoxin, AF64A have been applied to reduce cholinergic activity.

Although none of these animal models shows the neuropathological characteristics, such as senile plaques and NFT (see chapter 1), found in the brains of patients with AD, they have been widely used for assessing the validity of therapeutic interventions with cholinergic drugs (Murray and Fibiger, 1986; Itoh *et al.*, 1997).

Early work directed at lesioning the cholinergic neurons focused largely on the use of ibotenic acid to destroy cholinergic neurons of the nucleus basalis magoncellularis (NBM). Lesions of the NBM cause deficits on a broad range of tasks including passive avoidance (Matsuoka *et al*, 1992; Yamamoto *et al*, 1993; Yamamoto *et al*, 1994) and object discrimination learning (Ridley *et al*, 1985). Acquisition of a Morris water task can also be impaired (Berger-Sweeney *et al*, 1994).

Another area primarily focused upon lesions of the medial septal area (MS) that is located caudal to the NBM (McKinney *et al*, 1983). Like lesions of the NBM, lesions of the MS impair performance in some tests of memory (Decker at al, 1991; Miyamoto *et al*, 1987). The MS provides cholinergic input to the hippocampus (Amaral *et al*, 1985). These fibres from the medial septum and also the diagonal band of Broca reach the hippocampus via the fimbria fornix pathway and perforant pathway. It is commonly believed that the hippocampus is a central component of the memory related neural system (O'Keefe *et al*, 1978; Olton *et al*, 1979, Eichenbaum, 1992).

The hippocampal formation (which includes the hippocampus proper, the entorhinal cortex and the subiculum Amaral *et al*, 1989) is thought to be the site of initiation of Alzheimer's pathology.

Like all commonly used models of AD, lesions of the hippocampus readily produce deficits in many tasks, such as acquisition and performance on the Morris water task (Maier *et al*, 1990; Sutherland *et al*, 1986) and retention of passive avoidance (Olton, 1973). Hippocampal deafferentiation by damage to the entorhinal cortex can also cause cognitive dysfunctions (Rasmussen *et al*, 1989;Fugger *et al*, 1997; Eijkenboom *et al*; 2000). A role for the entorhinal cortex in modelling AD was based on post mortem studies, which revealed that at a very early stage of the disease the E.C. in the brains of Alzheimer's patients showed signs of degeneration and the extent of the degeneration was correlated with the memory deficits present during the early stage of AD (Hyman *et al*; Braak and Braak, 1991). In rats, bilateral damage to the E.C. resulted in learning and memory deficits due to disturbed hippocampal functioning (Rasmussen *et al*, 1989) and previous studies have shown that this deficit is sensitive to pharmacological enhancement, i.e. by aniracetam, an AMPA receptor agonist (Zajaczkowski, 1997) and memantine, an NMDA receptor antagonist. This thesis has shown EC lesions produce deficits in the Morris water escape task and can be alleviated by treatment with ChEI's.

Strain and Species comparisons

A comparative approach in which one or more species is considered is necessary for the decision to either reject or accept a specific model. This study looked at 2 different species of rodent to determine the effects of the cholinesterase inhibitors.

Some rat or mouse strains are more successful in certain tasks than others. Housing conditions, diet, biological rhythms, stress, route of administration, strain, age, handling and training parameters to name only a few can influence data significantly. Strain dependent effects may account for many conflicting results in the literature concerning cognitive performance (Andrews, 1996). Strains can differ in their pharmacodynamic and pharmacokinetic properties, which could possibly affect the outcome of the drug effects on behaviour. Therefore, well-defined, inbred strains can increase the reproducibility and predictability of results. Also, the environment in which strains are bred and kept must be highly standardised along with the testing area and procedure (Andrews, 1996) and the age at which the testing is performed (Meier, 1964).

Different strains must first be identified to be able to evaluate their influence on cognitive performance and drug evaluation. Small differences between strains were observed in the rat in chapter 9. The Hooded lister and the Harlan Wistar were compared in the two choice water escape task and it was found that the Hooded lister had faster escape latency. Because this is a measure of reference memory it is important to take into account this difference when doing further studies on spatial reference memory.

The reference compound

The compound used in this study was the second generation cholinesterase inhibitors metrifonate (See chapter 1). Previous work with metrifonate has shown that the compound to be an effective cognition enhancer in various animal models (Schmidt et al, 1997) and in patients with mild to moderate AD (Raskind *et al*, 1999;Gelina *et al*, 2000). During the course of this thesis, it was noticed that metrifonate produced very little effects in enhancing cognition in the tasks studied; moreover in some cases the compound impaired performance (See chapter 5 and 7).

The previously reported optimum dose range for metrifonate to produce positive effects on learning and memory was 10-30mg/Kg (van der Staay et al, 1996a), and particularly an effective dose was 12.5mg/kg (van der Staay et al, 1996b), however in this study, if positive results in the behavioural tasks were obtained, the optimum dose range was 30-60mg/kg. Another study had used a broad dose range, i.e.10-100mg/kg (Riekkinen et al, 1996) and had positive effects even with the higher dose range of 100mg/kg, with no suggestion of cholinergic side effects.

A previous report by van der Staay (1997) found that the performance of the Wistar strain of rat has shifted towards a poorer performance in the Morris water maze over a period of 71 weeks. This was conducted on aged (24 month old) rats, however it is likely that this shift in performance may also occur in young rats. In the van der Staay study it suggested a possible reason for the changes in performance between the two experiments could be due to genetic drift between the two different shipments of animals. Also, the possibility of biological variability or differences in sensitivity of the rats to the treatment with metrifonate could explain that a previous study found no impairments with the 100mg/kg dose whereas in this study it occurred regularly. This might suggest a narrowing of the therapeutic index of metrifonate in this strain of rat as well as a shift from the lower dose being optimum to a higher dose range.

Because of the inconsistent results of metrifonate in this study and because its clinical development has been discontinued, it is advisable to use other reference compounds. The compounds that could be used for future studies include donepezil or rivastigmine.

Rivastigmine (Exelon, ENA 713) is a phenyl-carbamate derivative with a centrally-acting and long lasting anti-acetylcholinesterase activity. In the rat, rivastigmine acts principally in the hippocampus and the cortex. It has been shown to improve the performance of scopolamine- treated and basal forebrain- lesioned rats, in the Morris water escape task and the step down avoidance task (Enz *et al,* 1989; Niigawa *et al,* 1995). This compound has been shown to be effective in patients with mild to moderate AD in a number of clinical trials (Corey-Bloom *et al,* 1998; Rösler *et al,* 1999) and is currently available in some European countries.

Donepezil (Aricept, E2020) is a piperidine compound that is a centrally acting cholinesterase inhibitor. Pharmacological studies *in vivo* and *in vitro* have shown that the compound acts reversibly and non-competitively (Rogers *et al*, 1991) with a high selectivity for acetylcholinesterase (>1250) over butyrylcholinesterase (Sherman, 1991). It produces long lasting brain acetylcholinesterase inhibition and increases the brain content of ACh *in vivo* (Yamanishi, *et al*, 1988). Donepezil has a long half-life of 70 hours, producing a steady state of inhibition after once daily administration within 14-21 days in humans (Rogers *et al*, 1998a and 1998b). This compound has been shown to be effective in patients with mild to moderate AD in a number of clinical trials (Rogers *et al*, 1998a and 1998b; Giacobini, 2000). In animal studies donepezil improved performance in the 8-arm radial maze in scopolamine treated rats and in rats with lesions of the nucleus basalis magnocellularis (nbm; see Rogers *et al*, 1991).

A reference compound needs to be effective across tasks, models and species. The cognitive enhancing properties of rivastigmine and donepezil have been observed in previous work (Enz *et al,* 1989; Niigawa *et al*, 1995; Rogers *et al*, 1991) and it is recommended these compounds are assessed as metrifonate has been in this thesis to determine its their effectiveness as reference compounds so that they can be used assess future models for AD

General considerations

D'Mello and Stecker (1996) introduced points that are needed to be considered when choosing an animal model and to assist in the design of new and improved animal model. Some of these features are discussed in reference to the experiments completed in this thesis.

- Paradigms need to be selected that are suitable for testing two or more species. Rodents are particularly well suited for cognition and ageing research, which is comparable to that of humans (Steckler & Muir, 1996). The comparative approach between species, for example the rat and the mouse help determine more about brain function and behaviour in order to extrapolate to the human. In this thesis the standard Morris water escape

task was used for the rat and the mouse (See chapter 5). The passive avoidance task (chapter 4) and the Two Choice water escape task (chapter 9) can also be adapted for the two species. It is also important to determine the efficacy on compounds in relation to different species, in the same task.

- Train a performance baseline that is neither too high or two low as drug or lesion induced changes in performance may be confounded by ceiling or floor effects. This was observed in normal animals in the Morris water escape task in chapter 5 in both experiment 1 and 2.

Select two or more paradigms each thought to measure the same cognitive process. This provides and estimate of the construct validity (See chapter 1) of an experimental approach, for example Morris water escape task (Chapter 3-5) and the Two Choice water Escape task (chapter 9) which both look at spatial navigation. However, it is important to determine as to whether the tasks use the same memory processes.

- Test a number of doses that range from just no effect to reliable motor disruption. This helps validate data that have no observable effects on motor function. i.e. in chapter 3, 10 30 or 60 mg/kg metrifonate were administered sub chronically and their effects determined in the Morris water escape task. 30 mg/kg metrifonate improved performance in this task whereas 10 mg/kg metrifonate had no effects and 60 mg/kg impaired performance.

- Where possible, measure mnemonic and non-mnemonic performance in the same animal and in the same task. i.e. in the two choice water escape task effects on both reference memory and attention could be observed. However, further study is required on this task.

- Finally, take into account the effects of individual animal variability. Ideally use a single subject repeated measures design. i.e. each animal serves as its own control. For example in chapter 8 each animal's peak time was measured on the day before and on the day of cholinesterase treatment.

Taking these considerations and the additional recommendations by D'Mello and Steckler (1996) and of Sarter *et al*, (1992), a broad characterisation of putative cognition enhances is recommended. As studied in this thesis, the efficacy of a test compound should be determined in a series of tests that cover a variety of cognitive processes (Maitre and Pepeu, 1989), the tests of which have previously been discussed.

Conclusions and Future study

There is no animal model available that can mimic all the cognitive, biochemical and histopathological abnormalities observed in patients with AD. However partial reproduction of AD neuropathology and cognitive deficits can be achieved. Future models that can be used to assess future cognition enhancers need to include the neuropathology of Alzheimer's disease. A growing number of studies have demonstrated that acute or continuous infusion of Aβ (See chapter 1) into the brain causes brain dysfunction as evidenced by neurodegeneration and an impairment of learning and memory (Pepeu *et al*, 1996; Yamada *et al*, 1999).

The effects of Aβ fragments on learning and memory were first examined in mice (Flood *et al*, 1991). Many subsequent studies have demonstrate that various Aβ fragments such as Aβ1-40, Aβ1-42 and Aβ25-35, cause learning and memory impairments in mice and rats (McDonald *et al*, 1994; Chen *et al*, 1996; Maurice *et al*, 1996, 1998; Pepeu *et al*, 1996; Harkany *et al*, 1998; Oka *et al*, 1999). This in vivo neurotoxicity produced by Aβ is potentiated by co-injection with ibotenic acid (Morimoto *et al*, 1998). From this it might be useful to suggest a combination of cholinergic lesions and Aβ administration, thus producing a more complex model in producing behavioural dysfunction and the underlying pathology related to AD. This then could be used to assess compounds for the treatment of AD.

Models employing transgenic mice might also be used to study future cognition enhancers. Transgenic mice overexpressing human APP751, which develop early AD like histopathology with diffuse deposits of Aβ and aberrant tau protein immunoreactivity in some cases (Higgins, *et al*, 1994), exhibit age-dependent deficits in spatial learning in a water maze and in spontaneous alternation behaviour in a Y-maze (Moran, *et al*, 1995).

There is no definitive behavioural model for AD. Although animal models discussed in this thesis, particularly EC lesions induce mnemonic deficits similar to those seen in AD, the relationship is less than perfect. Further testing in other spatial and non-spatial cognitive tasks might provide further insight into the character of the deficits. With greater specificity and sensitivity in the choice of task, i.e. improved validation of these tasks. Also, as discussed above employing animal models that integrate lesions, transgenic animals and/or pharmacological manipulation, could be important in expanding knowledge on the complex interactions that might exist between neuropathological changes and the functional consequences caused by brain damage. This combined approach needs to be studied to determine as to whether there is an increased validity of animal models of behavioural dysfunctions and therefore the evaluation of future cognition enhancers could become more effective.

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ABBREVIATIONS

Aß	ß Amyloid protein
Ach	Acetylcholine
Acetyl CoA	Acetyl coenzyme A
AChE	Acetylcholinesterase
AD	Alzheimer's Disease
ApoE	Apolipoprotein E
APP	ß amyloid Precursor Protein
BuChE	Butyrylcholinesterase
ChAT	Choline acetyltranferase
ChEI	Cholinesterase Inhibitors
CNS	Central Nervous System
E.C	Entohinal Cortex
FI	Fixed Interval
ITI	Intertrial Interval
MTP	Matching To Position
MWM	Morris Water Maze
NMTP	Non Matching To Position
PI	Peak interval
PS-1	Presenilin 1
PS-2	Presenilin 2
PT	Peak Time
PR	Peak Rate
SET	Scalar Expectancy Theory